



Molecular detection and characterisation of feline morbillivirus in domestic cats in Malaysia

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ARTICLE INFO

Keywords:

Feline morbillivirus (FeMV)
Molecular characterisation
N gene
L gene
Domestic cats
Malaysia

ABSTRACT

Feline morbillivirus (FeMV), a novel virus from the family of Paramyxoviridae, was first identified in stray cat populations. The objectives of the current study were to (i) determine the molecular prevalence of FeMV in Malaysia; (ii) identify risk factors associated with FeMV infection; and (iii) characterise any FeMV isolates by phylogenetic analyses. Molecular analysis utilising nested RT-PCR assay targeting the L gene of FeMV performed on either urine, blood and/or kidney samples collected from 208 cats in this study revealed 82 (39.4%) positive cats. FeMV-positive samples were obtained from 63/124 (50.8%) urine and 20/25 (80.0%) kidneys while all blood samples were negative for FeMV. In addition, from the 35 cats that had more than one type of samples collected (blood and urine; blood and kidney; blood, urine and kidney), only one cat had FeMV RNA in the urine and kidney samples. Risk factors such as gender, presence of kidney-associated symptoms and cat source were also investigated. Male cats had a higher risk ($p = 0.031$) of FeMV infection than females. In addition, no significant association ($p = 0.083$) was observed between the presence of kidney-associated symptoms with FeMV status. From the 82 positive samples, FeMV RNA was detected from 48/82 (58.5%) pet cats and 34/126 (27.0%) shelter cats ($p < 0.0001$). Partial L and N gene sequencing of the RT-PCR-positive samples showed 85–99% identity to the published FeMV sequences and it was significantly different from all other morbilliviruses. A phylogenetic analysis of the identified Malaysian FeMVs was performed with isolates from Japan, Thailand and China. Molecular characterisation revealed high relatedness of the Malaysian isolates with other Asian FeMVs, indicating that the virus had been circulating only within the region. Therefore, this study confirmed the existence of FeMV among domestic cats in Malaysia. The findings suggest further characterisation of the local isolates, including the whole genome sequencing and that studies at determining the direct consequences of FeMV infection in domestic cats are needed.

1. Introduction

Morbilliviruses are categorised in the order of Mononegavirales and the family of Paramyxoviridae. It is an enveloped single-stranded, negative-sense RNA virus that encodes a single envelope-associated matrix protein (M), two glycoproteins (hemagglutinin H and fusion protein F), two RNA-polymerase-associated proteins (phosphoprotein P and large protein L), and a nucleocapsid protein (N) that encapsulates the viral RNA (Sato et al., 2012). This group of virus causes significantly serious disease in their respective hosts: measles virus in human, rinderpest virus in cattle, phocine distemper virus in seals, peste des petits ruminant virus in sheep and goats and canine distemper virus (CDV) in several species of carnivores (Rima and Duprex, 2006). Each

morbillivirus infects a specific animal species due to the preferential use of species-specific signaling lymphocyte activation molecule (SLAM) as the receptor (Tatsuo et al., 2001). Until 2012, no morbillivirus-associated disease has been identified in domestic cats.

Although CDV infection has been reported in wild *felidae* such as lions, tigers and leopards, domestic cats could be seropositive with inapparent infection of CDV (Ikeda et al., 2001). In view of this phenomenon, a molecular epidemiology study was conducted, leading to the discovery and isolation of a proposed novel feline paramyxovirus in domestic cats in Hong Kong (Woo et al., 2012). It was then followed by the discovery of the feline paramyxovirus from other countries including Japan, Italy, Germany, USA, Turkey, Brazil and our Southeast Asian counterpart, Thailand (Darold et al., 2017; Furuya et al., 2014;

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<https://doi.org/10.1016/j.vetmic.2019.08.005>

Received 16 April 2019; Received in revised form 1 August 2019; Accepted 6 August 2019

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Lorusso et al., 2015; Sharp et al., 2016; Sieg et al., 2015; Yilmaz et al., 2017). The virus, named as feline morbillivirus (FeMV; initially abbreviated as FMoPV), has been identified having fewer than 80% nucleotide similarities to other known paramyxoviruses (Furuya et al., 2014; Woo et al., 2012). Phylogenetic analysis on six genes (*N*, *P*, *M*, *F*, *H* and *L* genes) in the first study of FeMV showed that all Hong Kong isolates were clustered together, forming a unique and distant cluster from the other morbilliviruses (Woo et al., 2012). Based on Park et al. (2014), there is no geographical relationship based on the FeMV phylogeny. Although phylogenetic analyses of identified FeMV in USA reported a global distribution of FeMV, the USA strain was closely related to Asian FeMV (Sharp et al., 2016).

Histological examinations and case-control studies linked the presence of FeMV with tubulointerstitial nephritis (TIN) in FeMV-positive cats (Woo et al., 2012). There were also higher FeMV-positive samples detected in kidney tissues compared to other type of samples, suggesting that the virus was nephrotropic (Furuya et al., 2014). Interestingly, a new feline paramyxovirus that shares 72–74% nucleotide homology to the bat and rodent paramyxovirus has been discovered (Sieg et al., 2015). A putative recombinant isolate of Japanese FeMV has been identified, involving the recombination of *F* and *H* genes between two virus isolates from Hong Kong and Japan (Park et al., 2014). A more recent study on molecular characterisation of FeMV suggested that the virus is to be divided into two genotypes based on the sequence homology with other known FeMVs (Sieg et al., 2018). Furthermore, the new genotype, FeMV-GT2 has been shown to infect lung epithelial, brain and immune cells other than the kidney epithelial cells (Sieg et al., 2019). As this novel virus may be more diverse in its pathogenicity than their counterpart, CDV, the presence of FeMV needs to be investigated and characterised. Further characterisation of FeMV isolated in Malaysia may potentially identify the origin of the virus, thus a better understanding on the evolution of FeMV. This study is a preliminary investigation of the existence of feline morbillivirus in Malaysia. The detection and molecular characterisation of this virus in domestic cats in Malaysia are important in order to examine the existence and impact of this virus to the feline population.

2. Materials and methods

2.1. Sample collection

The study was reviewed and approved by the Institution of Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia (UPM/IACUC/AUP-R057/2014). A total of 208 cats were sampled between 2014 and 2016 from veterinary clinics ($n = 82$) and animal shelters ($n = 126$) in Malaysia. The client-owned/pet cats were either presented to the veterinary clinics for annual health examination, neutering or health issues associated with kidney-related problem. The owner's consent was obtained prior to sample collection. Shelter cats were recruited as part of studies that investigated the prevalence of several other feline viruses. Therefore, not all type of samples (blood, urine or kidney) were collected from each cat. Either blood ($n = 61$), urine ($n = 90$) or kidney ($n = 22$) was obtained from 173 cats; both blood and urine samples were obtained from 32 cats; both blood and kidney samples were collected from one cat; and all three samples were collected from two cats. Kidney tissues collected through post-mortem examination were either from pet or shelter cats that died naturally or euthanised due to kidney-unrelated problems. Data such as the cat's age, sex and clinical findings were obtained for the pet cats; however, limited information could be obtained from shelter cats.

2.2. Inclusion criteria for kidney-related diseased cats

A total of 27 cats of which the serum urea and creatinine results were available was further analysed to determine the association of kidney-related disease with the presence of FeMV. Based on the serum

urea-creatinine results (International Renal Interest Society Guidelines), the cats were further sub-grouped into cats that had presence or absence of kidney-related disease. Cats having kidney-related disease were defined as having serum urea reading of more than 10.0 mmol/L and serum creatinine of more than 193 μ mol/L. Cats that had normal serum urea-creatinine level or either one elevated were grouped into cats that had absence of kidney-related disease. Previous history of renal/urinary system problems was also obtained.

2.3. Sample processing

Blood samples were collected via jugular/cephalic venipuncture, transferred into EDTA tubes and immediately subjected for RNA extraction. The urine samples were collected into a sterile conical tube either by manual compression or cystocentesis, followed by centrifugation at 1500 rpm for 10 min. to obtain the supernatant which were then stored at -20°C in RNeasy[®] solution (Qiagen, Hilden, Germany) at 1:1 ratio. Collected kidney samples were immediately placed into a sterile container containing RNeasy[®] solution for the viral RNA extraction. Prior to the extraction, kidneys were briefly dried on a filter paper and added into a phosphate-buffered saline solution at a concentration of approximately 1 g/mL. Then, the kidney samples were crushed into small pieces with a mortar and pestle. The mixture was then centrifuged at 2000 rpm for 10 min to remove large debris.

2.4. RNA extraction

The total RNA from blood samples and viral RNA from urine samples were extracted using QIAamp[®] RNA Blood Mini Kit (QIAGEN, Hilden, Germany) and QIAamp[®] Viral RNA Mini Kit (QIAGEN, Hilden, Germany), respectively. The total RNA was extracted directly from the kidney tissue lysates using the RNeasy[®] Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

2.5. Detection of feline morbillivirus targeting *L* gene by nested RT-PCR assay

FeMV detection was performed by amplifying 401-bp fragment of *L* gene of morbillivirus by using published conserved primers (Furuya et al., 2014). Reverse transcription (RT) was performed using SensiFAST cDNA Kit (Bioline, USA) with random hexamer and anchored oligo dT primers, according to the manufacturer's manual. This was followed by a two-step nested PCR assay targeting the *L* gene using MyTaq Mix (Bioline, USA) in My Cyclor[™] thermal cycler. The SS1 plasmid which contained the partial *L* gene (Furuya et al., 2015) was used as the positive control (courtesy of Dr. Tetsuya Furuya, Tokyo University of Agriculture and Technology) and sterile distilled water as the negative control. The amplified products were analysed on 1.5% agarose gels.

2.6. FeMV *N* gene amplification and sequencing of *N* and *L* genes

In order to further characterise the FeMV isolates obtained in Malaysia, random samples were chosen for a partial *N* gene amplification using a primer set (forward: 5'- AAG CTG TGA CTG CTC CAG AC -3'; reverse: 5'- GTA AGG TGC CAT TTC CCC CA -3') that was designed and optimised in the current study. The amplification was performed in a thermo cycler using the following conditions: 95°C for 1 min; 35 cycles of 95°C for 1 min, 51.7°C for 1 min and 72°C for 30 s; and a final extension at 72°C for 10 min. The PCR products were analysed on 1.5% agarose gels, and positive fragments of 398 bp were obtained. The PCR products were purified and sequenced on an ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems, USA) using the forward and reverse primers of *N* and *L* genes described in this study.

2.7. Phylogenetic analyses

The partial sequences of *NandL* genes of FeMV were aligned with previously reported FeMV using BioEdit and Molecular Evolutionary Genetics Analysis (MEGA) 7.0 software. Four other members of Morbilliviruses, canine distemper virus (CDV), dolphin morbillivirus (DMV), peste des petits ruminant virus (PPRV) and measles virus (MV) were retrieved from the GenBank® and used as outgroup. Phylogenetic analyses were carried out using Maximum Likelihood (ML) method with bootstrap values calculated from 1000 replicates between the feline morbillivirus and other known paramyxovirus based on the Tamura 3-parameter model via MEGA7 software (Tamura et al., 2013). Tamura 3-parameter model was identified as the best fitting model after a model test analysis (Tamura, 1992). The local FeMV sequences were compared with isolates from China, Japan, Italy, USA, Brazil and Germany as described in the GenBank database.

2.8. Data analyses

The association between FeMV status and gender, kidney-related disease or source of cats were analysed using chi-square test, with 95% confidence interval. All the analyses were performed using SPSS software, version 23.0 (SPSS, Chicago).

3. Results

3.1. Feline morbillivirus were detected from urine and kidney samples, but cats were not viremic at the time of sampling

The mean age of pet cats ($n = 82$) sampled in this study was 7.8 years (range: 1–20 years old) and 5.5 years for the 126 shelter cats, with age ranged between 1–14 years old. With regards to gender, a total of 100 (48.1%) male and 108 (51.9%) female cats were recruited in this study. From the 96 blood, 124 urine and 25 kidney samples screened for FeMV RNA targeting the highly conserved region of *L* gene, 63/124 (50.8%) urine and 20/25 (80.0%) kidney samples were positive (Fig. 1a). However, all 96 blood samples were negative for FeMV RNA indicating that the cats were not viremic at the time of sampling. From the total of 63 FeMV-positive urine samples, 32 samples originated from pet cats and the remaining were shelter cats. Meanwhile, FeMV-positive kidney samples were obtained from 16 pet cats and 4 shelter cats (Fig. 1b). In addition, from the 35 cats that had more than one type of samples (blood and urine; blood and kidney; blood, urine and kidney) collected, only one cat had both urine and kidney samples positive for FeMV RNA. Overall, the molecular prevalence of FeMV in this study was 39.4% (82/208).

3.2. Male and presence of kidney-related symptoms are associated with FeMV

Male cats were significantly associated with FeMV infection ($p = 0.031$) as 47/100 (47.0%) male cats were FeMV-positive compared to 35 out of 108 (32.4%) female cats (Table 1). Among the 27 cats of which the serum urea-creatinine results were obtained, only nine cats had elevated urea and creatinine level in the serum. Of these nine cats that had the presence of kidney-related symptoms, eight was found positive with FeMV by nested RT-PCR targeting *L* gene ($p = 0.083$). For the remaining 18 cats that had an absence of kidney-related symptoms, FeMV RNA was detected in ten cats. Interestingly, out of the total 82 FeMV-positive cats, 48 (58.5%) were pet cats and 34 (27.0%) were shelter cats ($p < 0.0001$).

3.3. FeMV Nucleocapsid (*N*) gene was amplified from randomly selected sample and confirmed by sequencing

FeMV-positive samples from the *L* gene screening were randomly

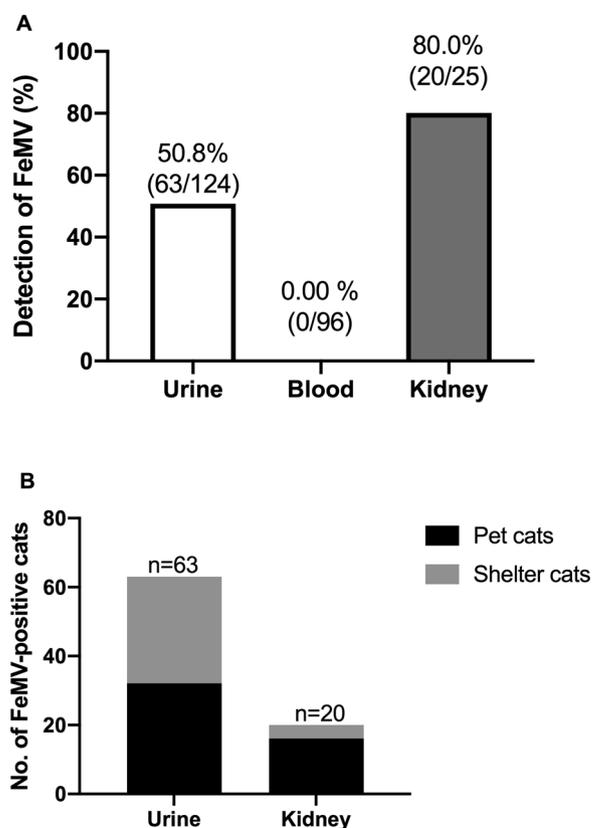


Fig. 1. A: Percentage of cats positive for FeMV in urine, blood and kidney samples. The number within the parentheses represents the number of positive samples out of the total number of samples tested. B: The distribution of FeMV-positive urine and kidney samples according to the type of cats.

selected for FeMV *N* gene PCR optimization, targeting the middle region of *N* gene. The primer used produced amplicons of the expected size of 398 bp (data not shown). A homology search of the generated sequences using BLAST analysis of the GenBank® database (National Center for Biotechnology Information, USA) confirmed that all five Malaysian isolates were FeMV. One of the positive isolates was later used as the positive control for further PCR screenings of the FeMV-positive samples, targeting the *N* gene. The detection rate of the *N* gene PCR assay for blood and urine samples were 0% and 32.4% (23/71), respectively. Interestingly, there were six urine samples that were positive for the nested RT-PCR targeting *L* gene but were found negative for the *N* gene RT-PCR.

3.4. Phylogenetic analysis of partial *L* and *N* genes revealed close-relatedness with isolates from Asian countries

Five FeMV-positive samples were randomly selected and sequenced for partial *L* and *N* genes. A BLAST analysis of the five cat samples revealed that the sequences possessed 85–99% nucleotide similarities with previously reported FeMV isolates. The highest similarity (99%) was with isolates reported from Asian countries (Thailand, Japan and Hong Kong) while the lowest similarity (85%) was with isolates reported from Germany that fell under genotype 2.

The sequences have been deposited under accession numbers KU646847- KU646856. The nucleotide sequences of partial *N* and *L* genes of the five Malaysian isolates and the outgroup sequences were aligned using Mega 7.0 software. Based on the phylogenetic tree of partial *L* (Fig. 2) and *N* genes (Fig. 3), all Malaysian isolates (UPM23; PCS139; UPM10; UPM53; RSS88) were clustered together and were closely related to the isolates reported from Hong Kong (M252A); Thailand (Thai-U16) and Japan (SS3, MiJP003, ChJP073, N153U,

Table 1
Descriptive characteristics of the FeMV-positive cats.

Factors	Total number of cats sampled (n)	Number of cats positive for FeMV (n)	Ratio	Percentage of FeMV-PCR positive cats	Odds ratio (95% CI)	P value
Gender						
Male	100	47	47/100	47.0		
Female	108	35	35/108	32.4	1.850 (1.054-3.247)	*0.031
Kidney-related symptoms						
Presence	9	8	8/9	88.9		
Absence	18	10	10/18	55.6	6.400 (0.656-62.40)	0.083
Source						
Pet cats	82	48	48/82	58.5		
Shelter cats	126	34	34/126	27.0	3.820 (2.118-6.889)	* < 0.0001

N040 K, N028 K, N141U, N010U). All previously reported FeMV formed a distinct cluster in the phylogenetic tree which was separated from the other members of the genus Morbillivirus.

4. Discussion

This study is the first in Malaysia to describe the existence of FeMV in domestic cats. The molecular prevalence of FeMV in the present study was 39.4% (82/208) which was higher than the reports from other countries, including Hong Kong (12.3%, 56/457) (Woo et al., 2012), Japan (6.1%, 5/82) (Furuya et al., 2014) and Turkey (5.4%, 6/111) (Yilmaz et al., 2017). The differences in the prevalence could be attributed to several factors, such as the variation of geographical location and the exposure to outdoor environment in both pet and shelter

cats. Furthermore, as the pathogenesis of FeMV is currently unclear, we could only suggest that the FeMV might have long incubation period in which the chronic shedding of the virus may facilitate transmission between cats especially for those that live in a multi-cat environment.

FeMV RNA could not be detected in the blood samples collected in this study, indicating that the viraemia might not be persistently observed in the infected cats or the viraemia duration was short, possibly due to efficient elimination by the host immune system (Lorusso et al., 2015; Yilmaz et al., 2017). The detection rate of FeMV was also found to be higher in the kidney (80.0%) than urine (50.8%). The result of our study was consistent with the report from Japan where the detection of FeMV was higher in kidney tissues compared to urine (Furuya et al., 2014). Various pathological changes such as interstitial cell infiltration, glomerulosclerosis, tubular atrophy and fibrosis have been significantly

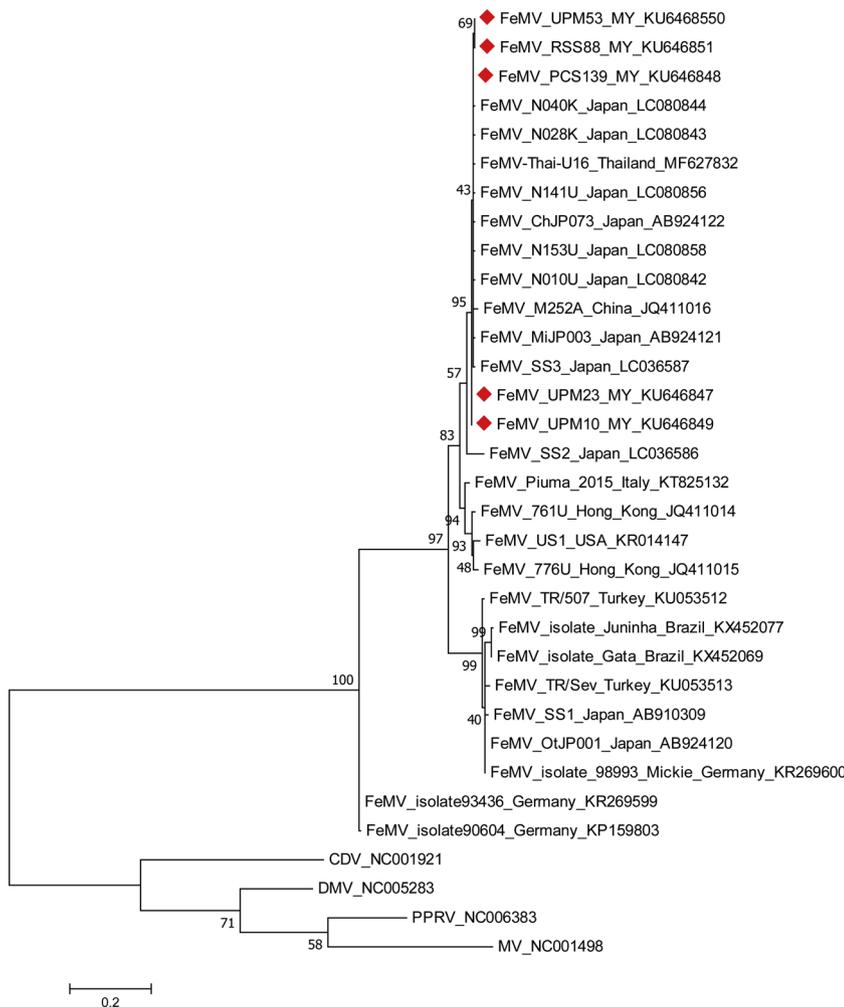


Fig. 2. Phylogenetic analysis of five feline morbillivirus isolates encoding partial L gene. Analyses were carried out using Maximum Likelihood method with bootstrap values calculated from 1000 replicates between the feline morbillivirus and other known paramyxoviruses based on the Tamura 3-parameter model by using MEGA 7. Four other members of Morbilliviruses [canine distemper virus (CDV), dolphin morbillivirus (DMV), peste des petits ruminant virus (PPRV) and measles virus (MV)] as outgroup were retrieved from the GenBank® and used as reference. The diamond (◆) indicates the five FeMV isolates used in this study. Bars indicate the estimated number of nt substitutions per site.

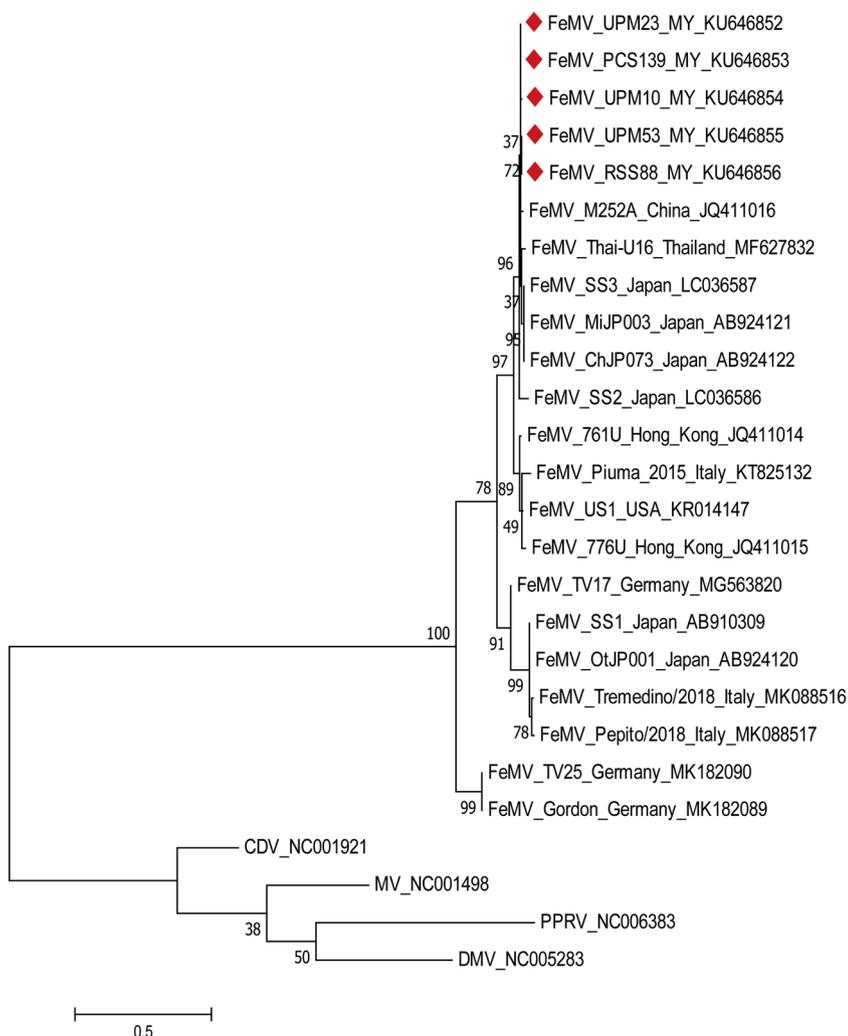


Fig. 3. Phylogenetic analysis of five feline morbillivirus isolates encoding partial *N* gene. Analyses were carried out using Maximum Likelihood method with bootstrap values calculated from 1000 replicates between the feline morbillivirus and other known paramyxoviruses based on the Tamura 3-parameter model by using MEGA 7. Four other members of Morbilliviruses [canine distemper virus (CDV), dolphin morbillivirus (DMV) peste des petits ruminant virus (PPRV) and measles virus (MV)] as outgroup were retrieved from the GenBank® and used as reference. The diamond (◆) indicates the five FeMV isolates used in this study. Bars indicate the estimated number of nt substitutions per site.

correlated with the presence of FeMV antigen (Sutummaporn et al., 2019). Furthermore, of the 45 cats that had more than one type of samples collected, only one cat was positive for both urine and kidney samples. Since this cat originated from a shelter, there was no further information that could be correlated with the findings. We could only speculate that the shedding of the virus could be due to the stage of FeMV infection when it tries to evade the host immune responses, representing a host-pathogen interaction (Ou et al., 2017).

It has also been reported that there is an association of feline morbillivirus with chronic kidney disease (CKD) characterised by tubulointerstitial nephritis (Furuya et al., 2014; Park et al., 2014; Sieg et al., 2015; Woo et al., 2012). In this study, there was no association ($p = 0.083$) between FeMV-positive cats with the presence of kidney/urinary-related disease as observed in other studies (McCallum et al., 2018; Park et al., 2016). This classification of kidney-diseased cats was made possible by the availability of serum urea and creatinine level and previous history of kidney-associated problems. Due to the limitation of the study, we did not obtain additional parameters that would enable us to stage the CKD in our sampled cats. To date, this novel virus cannot be conclusively associated with kidney disease as there are contrasting studies that found indirect association between these two variables (Woo et al., 2012; Furuya et al., 2014; Sieg et al., 2015). Therefore, prospectively studying a large number of cats with CKD together with the observation of pathological damage in the renal tissues and presence of viral RNA and acute/convalescent sera would be beneficial to establish the pathogenicity of FeMV. Several studies have also looked into other risk factors associated with feline morbillivirus infection such

as age and gender (Park et al., 2016; Sieg et al., 2015). In this study, a similar number of male to female (100:108) cats was recruited. There was an association between gender and FeMV infection ($P = 0.031$), implying that male has higher chance of getting FeMV infection compared to female cats. This could be due to the territorial fighting, aggressiveness and marking behaviour of male cats, increasing the risk of transmission between male cats as observed in other diseases (Goldkamp et al., 2008; Yamamoto et al., 1989).

In the current study, a primer set targeting the *N* gene region was designed and the assay was optimised using FeMV-positive samples based on the *L* gene screening. However, six urine samples that were previously found positive by *L* gene nested RT-PCR assay turned out negative for the FeMV *N* gene detection. It was suspected that sample degradation might have occurred during the storing period or the low viral load that was below the detection limit of the *N* gene conventional RT-PCR assay.

The phylogenetic analysis of the partial sequence of the *L* and *N* gene showed that the Malaysian isolates shared high similarity and clustered together with isolates from other Asian countries: Hong Kong (M252A); Thailand (Thai-U16) and Japan (SS3; MiJP003, ChJP073, N153U, N040 K, N028 K, N141U, N010U). These results suggested that the FeMV isolates in this study were circulating within the Asian region. Furthermore, the phylogenetic analysis suggested that all Malaysian FeMVs were divergent from the FeMVs isolated in USA, Italy, Brazil and Turkey as different lineages were observed. A whole genome sequence analysis would be beneficial to assess any recombination events that may have occurred as observed in other study (Park et al., 2014).

Furthermore, the availability of the sequences of novel viruses in public domain serves as a useful database for the rapid identification of emerging new diseases, such as in the case of MERS-CoV (Sridhar et al., 2015).

5. Conclusion

This study has confirmed the existence of FeMV in domestic cats in Malaysia with a molecular prevalence of 39.4%. The molecular characterisation revealed the high relatedness of the Malaysian isolates with other Asian FeMVs. Further studies are required to explore the full genome sequences and determine the pathogenesis of FeMV infection in domestic cats.

Funding

This work was supported by the Universiti Putra Malaysia [grant number: GP-IPM/2014/9434300].

Declaration of Competing Interest

The authors declare that they have no competing interests. The funding body has no specific role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

The authors would like to thank Dr. Tetsuya Furuya (Tokyo University of Agriculture and Technology) for providing the positive control for nested RT-PCR assay targeting the *L* gene. We are grateful to the participating veterinary clinics and animal shelters for their assistance in sample collection.

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