veterinary sciences

Brief Report

Development and Preliminary Analytical Evaluation of an *L* Gene-Targeted TaqMan Real-Time RT-PCR Assay for Feline Morbillivirus

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Simple Summary

Feline morbillivirus infects cats and has been associated with kidney disease, although its role in causing chronic kidney disease remains unclear. We developed a molecular detection test based primarily on viral sequences from China. Preliminary testing using prepared DNA and RNA standards showed consistent results within and between experimental runs at the concentrations tested. No cross-reaction was detected with five other viruses. The test may provide a preliminary analytical tool for feline morbillivirus research.

Abstract

Feline morbillivirus (FeMV) has been associated with feline renal disease, highlighting the need for molecular tools to investigate its epidemiology and clinical significance. This study developed an *L* gene-targeted two-step TaqMan reverse transcription–quantitative polymerase chain reaction (RT-qPCR) assay using Chinese FeMV sequences as the primary design reference, supplemented by sequences from other geographic regions. Analytical performance was evaluated using plasmid DNA standards and in vitro-transcribed RNA standards. Plasmid DNA standards were tested directly by qPCR, whereas in vitro-transcribed RNA standards underwent reverse transcription before qPCR. The DNA- and RNA-derived standard curves yielded coefficients of determination (R^2) of 0.9948 and 0.9959, with estimated amplification efficiencies of 91.0% and 106.3%, respectively. The lowest tested concentrations detected were 7.94×10^1 copies/μL for plasmid DNA and 8.53×10^3 copies/μL for in vitro-transcribed RNA, with corresponding mean quantification cycle values of 37.61 ± 0.27 and 37.645 ± 0.637, respectively. Separate precision experiments showed consistent intra- and inter-assay performance at the tested concentrations. In the specificity assessment, recombinant FeMV served as the positive control. No amplification was observed within other tested viruses. These findings establish preliminary analytical performance with the selected laboratory materials under the tested conditions.

Keywords: feline morbillivirus; *L* gene; TaqMan; RT-qPCR

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1. Introduction

Feline morbillivirus (FeMV), a member of the genus Morbillivirus within the family Paramyxoviridae, was first reported in domestic cats in Hong Kong and mainland China in 2012 and has subsequently been detected in multiple geographic regions [1,2]. Although FeMV infection has been associated with feline renal disease, its causal role in chronic kidney disease remains uncertain. Molecular detection is therefore important for investigating its distribution and potential clinical significance.

The FeMV genome comprises six genes arranged in the order 3′-N-P/V/C-M-F-H-L-5′, which collectively encode six structural and two nonstructural proteins [1]. The *L* gene encodes the large polymerase protein, which associates with the phosphoprotein (P) to form the RNA-dependent RNA polymerase complex responsible for viral RNA synthesis [3,4]. Conserved regions within the *L* gene have been successfully targeted in conventional and quantitative RT-PCR assays, providing an established basis for its use in molecular detection [1,3,5]. Nevertheless, conservation within individual primer- and probe-binding sites requires evaluation across the sequences of interest [6].

Several reverse transcription–quantitative PCR (RT-qPCR) assays targeting the *L*, *N*, or *P*/*V*/*C* regions have been developed for FeMV detection [3,5,7]. Furuya et al. established an L gene-targeted assay using sequences from six Japanese strains and demonstrated its applicability to feline urine samples [3]. However, the authors acknowledged that their primer–probe set might not detect some subsequently reported strains and highlighted the need to incorporate additional sequence information into assay design. Consistent with this concern, our comparative alignment identified mismatches between the published primer–probe set and several of the examined FeMV sequences. The alternative set designed in this study showed fewer mismatches for some sequences, although mismatches remained in others. The previously published *L* gene-targeted assay assessed analytical sensitivity using plasmid DNA standards [3]. Although that study also tested clinical RNA samples, its DNA-based calibration did not incorporate the reverse-transcription step. Evaluation using both DNA and in vitro-transcribed RNA standards can therefore provide complementary information about qPCR amplification and the combined reverse-transcription and amplification workflow.

In this study, we designed an alternative *L* gene-targeted primer–probe set using Chinese FeMV sequences as the primary design reference, together with sequences from other geographic regions to assess variation within the selected binding sites. The resulting two-step TaqMan RT-qPCR assay was preliminarily evaluated using plasmid DNA standards, tested directly by qPCR and in vitro-transcribed RNA standards analyzed following reverse transcription. This study aimed to characterize the assay's preliminary analytical performance under laboratory conditions and provide a basis for evaluating its potential as a complementary approach to existing methods for FeMV detection.

2. Materials and Methods

2.1. Construction of a Full-Length Antigenomic cDNA Clone and Recovery of Recombinant FeMV

In the absence of FeMV-positive clinical specimens, a reverse genetics system was established to recover recombinant FeMV. Full-length antigenomic cDNA corresponding to the FeMV reference genome (GenBank accession no. JQ411014) was chemically synthesized by Ruibo Xingke Biotechnology Co., Ltd. (Harbin, China) and assembled from six fragments into the pBluescript SK II (+) backbone named pBS-FeMV. The construct contained a T7 promoter and a hammerhead ribozyme upstream of the antigenomic sequence, with the δ and Φ regulatory elements positioned downstream. Three helper plasmids, pCAGGS-N, pCAGGS-P, and pCAGGS-L, were constructed to express the viral

nucleoprotein, phosphoprotein, and large polymerase protein, respectively. Stable BHK-T7 and CRFK-FeCD150 cell lines expressing T7 RNA polymerase and feline CD150, respectively, were generated by lentiviral transduction (Figure A1). The antigenomic construct and helper plasmids were cotransfected into BHK-T7 cells. Culture supernatants were collected at 72 h post-transfection and transferred to CRFK-FeCD150 cells for virus propagation. Syncytium formation was subsequently observed, and FeMV-specific RNA was detected by RT-PCR following passage.

2.2. TaqMan-Based RT-qPCR Assay

FeMV sequences from China and other countries were retrieved from GenBank under accession numbers JQ411014, JQ411015, JQ411016, AB910309, AB924121, AB924122, KP014147, KT825132, MG563820, MF627832, MN164531, MN164532, and OR086108. The sequences were aligned using the ClustalW algorithm implemented in MEGA (version 12.0.14) to identify a conserved target region within the *L* gene. The primer–probe set was designed to generate a 177 bp amplicon. The forward primer was 5′-TGTTCAGGGCCAGAGAGAAT-3′, the reverse primer was 5′-CTTGAGGTCTCAATGTGGCT-3′, and the probe was 5′-CCAACCCACGTAGACTCTGGTTTACCA-3′. Alignment of primer and probe sequences in this study and the published literature [3] is shown in Figure A2 with corresponding regions of FeMV genomes. The FeMV TaqMan probe was labeled with a 5′ 6-FAM reporter, an internal ZEN quencher, and a 3′ BHQ1 quencher. Two-step real-time RT-PCR was performed using a QuantStudio 5 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA), utilizing Probe qPCR Mix MultiPlus (Takara, Shiga, Japan), following the manufacturer's suggestion. Briefly, each 20 μL reaction contained 10 μL of probe qPCR Mix MultiPlus, 0.4 μL each of the forward and reverse primers (5 μM), 0.8 μL of probe (5 μM), 2 μL of template, 0.4 μL of ROX Reference Dye (50 X), and 6 μL nuclease free water to the final volume. Thermal cycling consisted of an initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. All samples were tested in triplicates. Amplification data were analyzed using QuantStudio™ Design & Analysis Software v1.3.1. Baseline correction was performed using automatic baseline determination, and the fluorescence threshold was set automatically. Cq values were determined using the Baseline Threshold algorithm.

2.3. Preparation of the Plasmid Standard

The recombinant plasmid PCAGGS-FeMV L, containing the FeMV *L* gene (total plasmid length: 11,488 bp), was constructed and used as the standard template for the absolute quantification of the qPCR assay. The plasmid DNA concentration was measured by UV spectrophotometry at 260 nm, using a microvolume spectrophotometer (NanoPhotometer NP80-Touch, Implen GmbH, Munich, Germany). The A_{260}/A_{280} and A_{260}/A_{230} ratios were 1.851 and 2.106, respectively. The plasmid was used without linearization to prepare the DNA standard dilution series. The concentration of plasmid was adjusted to a stock concentration of 1 μg/μL. The copy number of the plasmid standard was calculated using the following formula:

$$\text{“Copies/μL”} = (\text{“Concentration (ng/μL)”} \times 6.022 \times 10^{23})/(\text{“Plasmid Size (bp)”} \times 660 \times 10^{9})$$

The calculated stock concentration was approximately 7.94×10^{10} copies/μL. Tenfold serial dilutions were prepared in nuclease-free water, beginning at 7.94×10^{9} copies/μL and continuing down to 7.94×10^{1} copies/μL. The undiluted stock and the serial dilutions were tested in triplicate, giving an overall tested range of 7.94×10^{1}–7.94×10^{10} copies/μL. These standards were used directly as DNA templates to assess qPCR detection sensitivity

and generate a standard curve by plotting Cq values against the $\log_{10}$-transformed plasmid copy concentrations. All reactions were conducted in triplicate.

2.4. Preparation of RNA Standard

The pBS-FeMV plasmid, containing the full-length FeMV genome sequence downstream of a T7 promoter, was used to generate the RNA standard. Following plasmid linearization, in vitro transcription was performed using the HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs, Ipswich, MA, USA), according to the manufacturer's instructions. The transcript was treated with DNase to remove residual template DNA, purified by phenol–chloroform extraction, and quantified. The RNA concentration was 75 ng/μL, corresponding to a calculated concentration of 8.53×10^9 copies/μL. Tenfold serial dilutions of the complementary RNA (cRNA) standard were prepared and reverse-transcribed using HiScript II Q Select RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China). For each dilution, 2 μL of RNA was reverse-transcribed in a total volume of 20 μL. The resulting cDNA was used without dilution, and 2 μL was added to each qPCR reaction. Each reaction therefore represented 0.2 μL of the original RNA dilution.

2.5. Specificity and Sensitivity

The analytical specificity of the assay was evaluated using nucleic acids extracted from feline calicivirus (FCV), feline coronavirus (FCoV), feline herpesvirus (FHV), feline panleukopenia virus (FPV), and canine distemper virus (CDV). Viral RNA or DNA was extracted using the UElandy® UE Body Fluid Viral DNA/RNA Miniprep Kit (UElandy Biotech, Suzhou, China). RNA samples were reverse-transcribed into cDNA, whereas DNA samples were used directly for qPCR. Recombinant FeMV recovered by reverse genetics and subsequently passaged in CRFK cells served as the positive control. All reactions were performed in triplicate.

Analytical sensitivity was evaluated using tenfold serial dilutions of the plasmid DNA and cRNA standards. Serial dilutions were prepared by adding 10 μL of each standard to 90 μL of nuclease-free water, followed by vortexing for at least 10 s before preparing the next dilution. Plasmid DNA dilutions were analyzed directly by qPCR, whereas cRNA dilutions were reverse-transcribed before qPCR analysis. Each dilution was tested in triplicate to determine the lowest tested concentration detected under the experimental conditions.

2.6. Reproducibility

In order to assess the reproducibility of the developed assay, three samples of different concentrations for the cRNA (8.53×10^7, 8.53×10^6 and 8.53×10^5 copies/μL) and plasmid DNA (7.94×10^7, 7.94×10^6 and 7.94×10^5 copies/μL) were selected, respectively. All experiments were performed under consistent laboratory conditions using the same reagents, instrument, and operator. For intra-assay assessment, each concentration from the same batch of diluted standards was tested in triplicate per run. For inter-assay assessment, standards at each concentration from three independently prepared batches were tested in three separate runs. Each cRNA replicate underwent an independent reverse-transcription reaction followed by qPCR, whereas plasmid DNA replicates were analyzed directly by qPCR. Mean Cq values, standard deviations (SDs), and coefficients of variation (CVs) were calculated by using Microsoft Excel Software (Version 2021, USA) for the intra- and inter-assay assessments.

3. Results

3.1. Selection of Primer and Probe Concentrations for the FeMV L Gene-Targeted TaqMan RT-qPCR Assay

Primer and probe concentrations were evaluated based on Cq values (Tables A1 and A2). Among the primer working concentrations tested, 5 µM yielded the lowest mean Cq value (11.417). For the probe, working concentrations of 2.5, 5, and 10 µM yielded similar mean Cq values of 12.800, 12.762, and 12.808, respectively; 5 µM was selected for subsequent experiments. The resulting 20 µL qPCR mixture contained 0.4 µL of each primer (5 µM) and 0.8 µL of probe (5 µM), corresponding to final concentrations of 0.1 µM for each primer and 0.2 µM for the probe.

3.2. Analytical Sensitivity of the FeMV L Gene-Targeted RT-qPCR Assay

The plasmid standard had a concentration of 1 µg/µL, equivalent to 7.94×10^{10} copies/µL. Tenfold serial dilutions ranging from 7.94×10^{1} to 7.94×10^{10} copies/µL were tested to evaluated qPCR sensitivity. The lowest tested concentration detected was 7.94×10^{1} copies/µL, corresponding to a nominal input of 158.8 copies per qPCR reaction, with a mean Cq value of 37.61 ± 0.27 (Figure 1A). The plasmid-based standard cure yielded a slope of −3.5583 and an R^2 of 0.9948, corresponding to an amplification efficiency of 91.0% (Figure 1B).

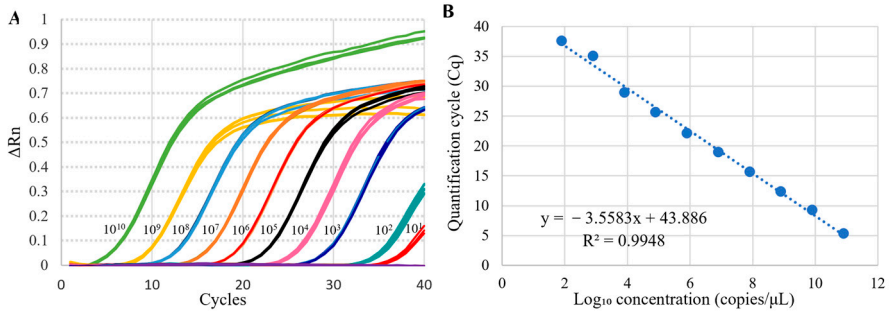

Figure 1. Amplification curves and plasmid-based standard curve of the FeMV *L* gene-targeted TaqMan qPCR assay. (**A**) Amplification curves obtained using tenfold serial dilutions of the plasmid DNA standard ranging from 7.94×10^{1} to 7.94×10^{10} copies/µL. Numbers indicate template concentrations in copies/µL. Each dilution was tested in triplicate. (**B**) Standard curve showing Cq values plotted against $\log_{10}$-transformed plasmid concentrations from 7.94×10^{1} to 7.94×10^{10} copies/µL.

The cRNA standard had a concentration of 75 ng/µL, corresponding to a calculated concentration of 8.53×10^{9} copies/µL. Tenfold serial dilutions ranging from 8.53×10^{1} to 8.53×10^{9} copies/µL were reverse-transcribed and subsequently analyzed by qPCR. No amplification was detected in the no-RT control using the DNase-treated, purified RNA standard, consistent with the no-template control and indicating no detectable residual amplifiable DNA under the tested conditions (Figure A4). The lowest tested RNA concentration yielding amplification was 8.53×10^{3} copies/µL, with a mean Cq of 37.645 ± 0.637, corresponding to a nominal input of 1.71×10^{3} RNA copies per qPCR reaction (Figure 2A). The RNA-based standard curve yielded a slope of −3.1796 and an R2 of 0.9959, corresponding to an amplification efficiency of 106.3% estimated across the combined reverse-transcription and qPCR workflow (Figure 2B), which was within the commonly accepted range.

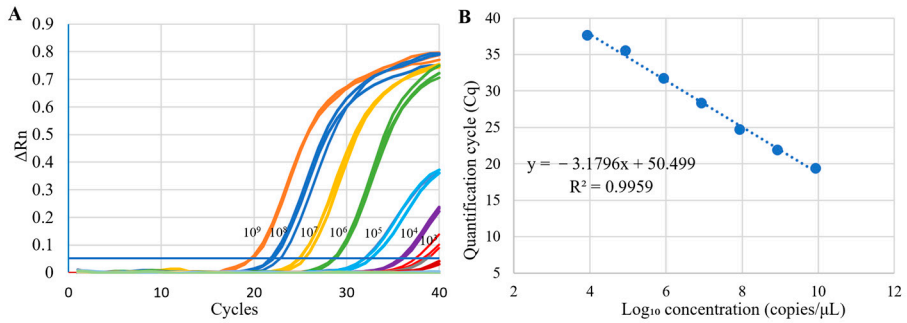

Figure 2. Amplification curves and RNA-based standard curve of the FeMV *L* gene-targeted two-step TaqMan RT-qPCR assay. (**A**) Amplification curves obtained from tenfold serial dilutions of the in vitro-transcribed RNA standard ranging from 8.53×10^1 to 8.53×10^9 copies/µL following reverse transcription. Numbers indicate RNA concentrations in copies/µL before reverse transcription. Each RNA dilution was reverse-transcribed once, and the resulting cDNA was analyzed in three technical qPCR replicates. These replicates therefore assess qPCR variability within a single reverse-transcription preparation. (**B**) RNA-based calibration curve showing Cq values plotted against $\log_{10}$-transformed RNA concentrations from 8.53×10^3 to 8.53×10^9 copies/µL before reverse transcription.

3.3. Analytical Specificity of TaqMan-Based RT-qPCR Targeting L Gene of FeMV

Cross-reactivity was assessed using nucleic acid preparations from FPV, FHV, FCV, FCoV and CDV. Recombinant FeMV recovered by reverse genetics and subsequently passaged in CRFK cells served as the positive control (Figure A3), yielding a mean Cq value of 29.53 ± 0.84 (Figure 3). No amplification was observed with any of the five non-target viruses or the no-template control (NTC). The presence of the respective viral targets in the comparator preparations was confirmed by PCR or RT-PCR, as appropriate. These results indicate no detectable cross-reactivity within the tested viral panel.

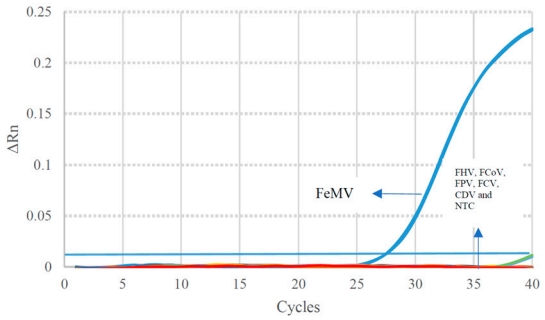

Figure 3. Analytical specificity of the FeMV *L* gene-targeted TaqMan RT-qPCR assay. Recombinant FeMV served as the positive control and produced amplification curves. No amplification above the threshold was detected with FPV, FHV, FCoV, FCV, CDV or NTC. The horizontal blue line represents the fluorescence threshold. All reactions were performed in triplicate. The extracted preparations had concentrations of 30–70 ng/µL. For RNA preparations, 2 µL (60–140 ng) was reverse-transcribed in a total volume of 20 µL. The resulting cDNA was used without further dilution, and 2 µL was added to each qPCR reaction, corresponding to 6–14 ng of input RNA equivalents per reaction, whereas DNA preparations were tested directly.

3.4. Reproducibility of TaqMan-Based RT-qPCR Targeting L Gene of FeMV

The reproducibility of the RT-qPCR was determined by the intra- and inter-assay evaluation of the Cq value using three samples with different concentrations for the plasmid DNA (7.94×10^7, 7.94×10^6 and 7.94×10^5 copies/µL) and cRNA (8.53×10^7, 8.53×10^6 and 8.53×10^5 copies/µL), respectively. The intra-assay was analyzed by using three samples in triplicates per run, whereas for the inter-assay assessment, the templates were

amplified in triplicates in three different consecutive runs. For plasmid DNA, the intra-assay SD and CV of Cq values ranged from 0.10 to 0.11 and from 0.47 to 0.68%, respectively. The corresponding inter-assay values ranged from 0.17 to 0.88 and 1.05 to 3.65%, respectively (Table 1). For cRNA, the intra-assay SD and CV ranged from 0.23 to 0.58 and 0.98 to 2.05%, respectively; whereas inter-assay SD and CV ranged from 0.72 to 0.98 and 2.26 to 3.49%, respectively (Table 2).

Table 1. Intra- and inter-assay variation for plasmid DNA standards.

Intra-Assay	Cq value			Mean Cq	S.D.	CV%
Standard Con	Replicate 1	Replicate 2	Replicate 3			
7.94×10^7	16.33	16.44	16.55	16.44	0.11	0.68
7.94×10^6	19.97	19.76	19.85	19.86	0.10	0.51
7.94×10^5	23.53	23.31	23.45	23.43	0.11	0.47
Inter-Assay	Cq value			Mean Cq	S.D.	CV%
Standard Con	Assay 1	Assay 2	Assay 3			
7.94×10^7	16.09	16.43	16.28	16.27	0.17	1.05
7.94×10^6	20.43	20.06	19.64	20.04	0.39	1.97
7.94×10^5	24.98	23.73	23.30	24.00	0.88	3.65

Table 2. Intra- and inter-assay variation for cRNA standards.

Intra-Assay	Cq value			Mean Cq	S.D.	CV%
Standard Con	Replicate 1	Replicate 2	Replicate 3			
8.53×10^7	23.68	23.54	23.23	23.48	0.23	0.98
8.53×10^6	27.41	28.52	28.23	28.06	0.58	2.05
8.53×10^5	31.51	31.14	32.19	31.61	0.54	1.69
Inter-Assay	Cq value			Mean Cq	S.D.	CV%
Standard Con	Assay 1	Assay 2	Assay 3			
8.53×10^7	24.74	23.39	24.84	24.32	0.81	3.32
8.53×10^6	28.36	27.17	29.12	28.21	0.98	3.49
8.53×10^5	31.74	32.44	31.01	31.73	0.72	2.26

4. Discussion

This study developed an alternative *L* gene-targeted TaqMan RT-qPCR assay for FeMV and evaluated its preliminary analytic performance plasmid DNA and in vitro-transcribed RNA standards. The assay exhibited consistent intra- and inter-assay performance at the tested concentrations and no detectable cross-reactivity within the tested panel, which included CDV. Together with comparative sequence analysis, these findings support its potential as a complementary molecular tool for FeMV research. Its strain coverage and clinical performance remain to be established through direct comparison with existing assays and evaluation of naturally infected specimens.

The assay was designed with particular attention to sequence compatibility with FeMV strains from China. Comparative alignment revealed differences in matching patterns of the present and previously published primer–probe sets. The present showed fewer mismatched for some sequences, where the published set matched Japanese strains more closely [3]. Primer–template mismatches can affect amplification and quantification, supporting the assessment of binding site conservation when assays are applied to additional viral sequences [7]. Unlike the previously published *L* gene-targeted assay, for which the reported analytic detection threshold was established using DNA standards [3], the present study evaluated both plasmid DNA and in vitro-transcribed RNA, extending the assessment to include reverse transcription. The assay is a complement of published

methods. The lowest tested concentrations detected were 7.94×10^1 copies/μL for plasmid DNA. This value cannot be directly compared with the previously reported threshold of fewer than 10 copies per reaction without accounting for the template input volume. At the least, our method shows the low detection concentration. However, the plasmid DNA standard was not linearized before qPCR, which may introduce conformation-related bias into quantification despite the high R^2 value. This effect was not assessed and should be examined in future validation [8].

The lowest tested RNA concentration yielding amplification was 8.53×10^3 copies/μL, numerically lower than the 1.0×10^4 copies/μL reported for the *N* gene-targeted assay [9]. However, differences in standard preparation, template input, reverse-transcription conditions, and endpoint determination preclude a direct conclusion of improved sensitivity. The RNA-based value represents an apparent amplification efficiency estimated across the combined reverse-transcription and qPCR workflow, rather than a direct measure of reverse-transcription efficiency. Neither standard accounts for nucleic acid extraction from clinical specimens. Consistent with MIQE 2.0 recommendations, further evaluation should distinguish detection from quantification limits and establish a detection probability near the analytical endpoint [10].

The absence of amplification with FPV, FHV, FCV, FCoV and CDV supports analytical specificity within the tested panel. Inclusion of CDV strengthens this assessment by adding a related morbillivirus. Recombinant FeMV provided a positive-control source in the absence of clinical material, but material derived from a single reference genome cannot represent the genetic diversity of circulating viruses. Previous detection of divergent FeMV sequences highlights the importance of evaluating strain coverage beyond the reference sequence [11].

The principal remaining limitation is the absence of naturally infected clinical specimens. Unlike the present preliminary evaluation, the assays reported by Furuya et al. and De Luca et al. included testing of clinical samples, providing evidence of applicability to biological matrices [3,5]. The P/V/C-targeted assay of De Luca et al. also represents an established alternative genomic target. Neither synthetic standard used in the present study accounts for extraction losses, sample-associated inhibition, or variation in viral RNA integrity. Clinical validation should therefore assess relevant specimen types and include direct comparison with an established assay using the same samples. Together with expanded strain testing, such work will determine the practical value of the present assay as a complementary tool for FeMV investigation. Its current contribution is a defined alternative design and preliminary analytical dataset that support this further evaluation.

5. Conclusions

This study developed and preliminarily evaluated an FeMV *L* gene-targeted two-step TaqMan RT-qPCR assay using Chinese FeMV sequences as the primary design reference. Evaluation with plasmid DNA and in vitro-transcribed RNA standards demonstrated linear calibration relationships and consistent intra- and inter-assay performance at the tested concentrations. No detectable cross-reactivity was observed within the tested viral panel, including CDV. These findings establish preliminary analytical performance with the selected laboratory materials under the tested conditions. Detection reliability across diverse FeMV strains, performance in naturally infected specimens, and any advantage over established assays remain to be determined.

Author Contributions: Conceptualization, H.J. and H.G.; methodology, C.X. and S.K.S.; software, C.X. and S.K.S.; validation, S.K.S., C.X. , H.J. and H.G.; formal analysis, C.X. and S.K.S.; investigation, C.X. and S.K.S.; resources, C.X. and S.K.S.; data curation, S.K.S. and H.G.; writing—original draft

preparation, S.K.S. and H.G.; writing—review and editing, H.G.; visualization, C.X. and S.K.S.; supervision, H.G. and H.J.; project administration, H.J.; funding acquisition, H.J. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

FeMV	Feline Morbillivirus
RT-qPCR	Reverse Transcription–quantitative polymerase chain reaction
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
cDNA	Complementary DNA
cRNA	Complementary RNA
FPV	Feline panleukopenia virus
FHV	Feline herpesvirus
FCV	Feline calicivirus
FCoV	Feline coronavirus
CDV	Canine distemper virus
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
N	Nucleoprotein
P	Phosphoprotein
L	Large polymerase protein
BHK	Baby hamster kidney
CRFK	Crandell–Rees feline kidney

Appendix A

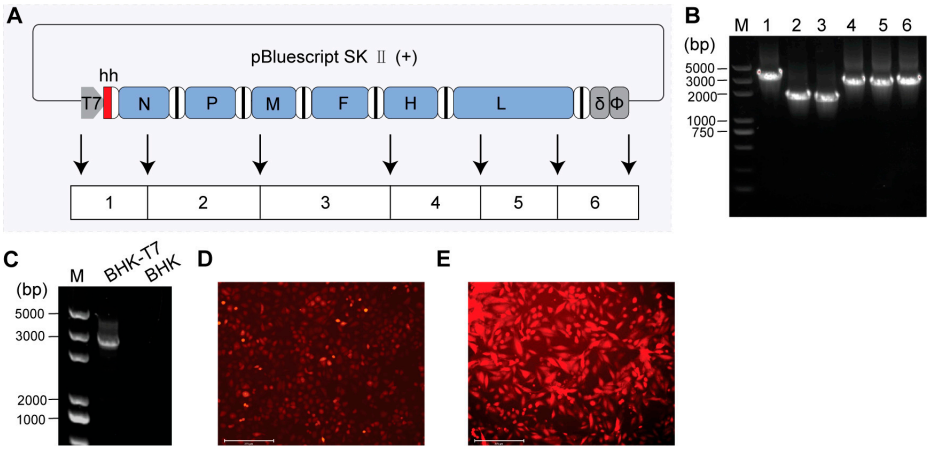

Figure A1. Construction of the FeMV reverse-genetics plasmids and characterization of engineered cell lines. (**A**) Schematic representation of the full-length FeMV antigenomic cDNA construct assembled from six fragments in the pBluescript SK II (+) backbone. The construct contains an upstream T7 promoter and hammerhead ribozyme (hh), with downstream elements designated δ and Φ. (**B**) PCR verification of the six inserted fragments; lanes 1–6 correspond to fragments 1–6 shown in panel A. (**C**) PCR analysis of BHK-T7 cells, with parental BHK cells included as a control. (**D**,**E**) Representative mCherry fluorescence images of BHK-T7 and CRFK-FeCD150 cells, respectively. M, DNA size marker; sizes are indicated in base pairs.

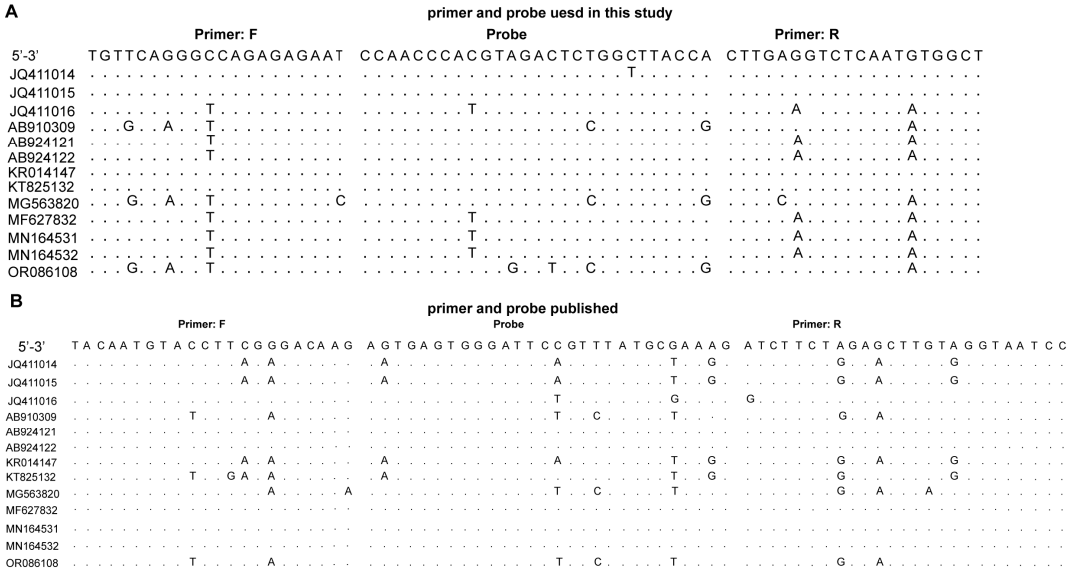

Figure A2. Alignment of primer and probe sequences with corresponding regions of feline morbillivirus (FeMV) genomes. Panels show the primer–probe set developed in this study (**A**) and a previously published set (**B**) [3]. The top row displays the forward primer, probe, and reverse primer sequences, each in the 5′–3′ direction. Dots indicate nucleotide identity with the respective primer or probe, whereas letters indicate mismatches. GenBank accession numbers identify the FeMV sequences included in the analysis. F, forward primer; R, reverse primer.

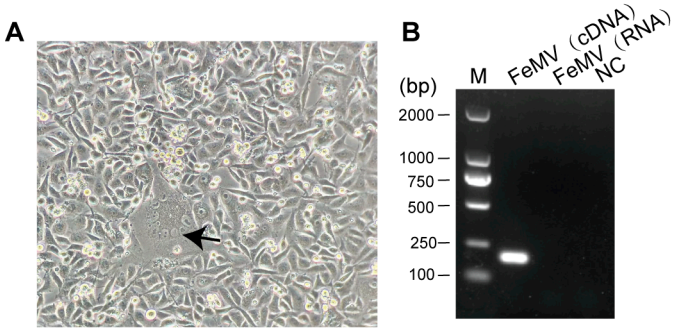

Figure A3. Characterization of recombinant feline morbillivirus (FeMV). (**A**) Cytopathic effects in CRFK-FeCD150 cells following inoculation with recombinant FeMV. The arrow indicates syncytium formation. (**B**) Agarose gel electrophoresis of RT-PCR products obtained from the recombinant FeMV (7th generation). M, DNA size marker; FeMV (cDNA), cDNA from reverse transcription of recombinant FeMV RNA; FeMV (RNA), RNA extracted from recombinant FeMV; NC, negative control. Marker sizes are indicated in base pairs (bp).

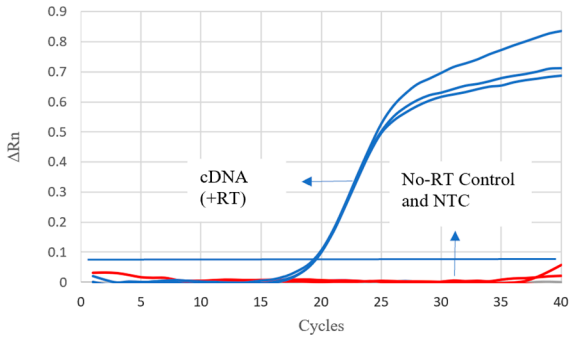

Figure A4. Assessment of residual amplifiable DNA in the RNA standard using a no-reverse-transcription control. The DNase-treated, purified in vitro-transcribed RNA standard was analyzed by qPCR following reverse transcription (+RT) or without reverse transcription (no-RT). Amplification was observed in the +RT reactions, whereas no detectable amplification was observed in the no-RT control or the no-template control (NTC) during 40 cycles. The horizontal line indicates the fluorescence threshold. ΔRn, baseline-corrected normalized fluorescence.

Appendix B

Table A1. Optimization of primer.

Primer Con (μM)	Ct-Value			Ct-Value (AV)
10	11.963	11.602	11.669	11.744
5	11.645	11.008	11.598	11.417
2.5	12.119	12.428	12.032	12.193
1.25	14.477	15.156	14.917	14.850
0.625	18.753	18.644	19.250	18.882
0.315	26.918	30.645	30.629	29.397

Note: Con, concentration; Ct, Cycle Threshold; AV, average.

Table A2. Optimization of probe.

Probe Con (μM)	Ct-Value			Ct-Value (AV)
10	12.506	13.023	12.896	12.808
5	12.754	12.724	12.810	12.762

2.5	12.778	12.921	12.702	12.800
1.25	14.556	14.817	15.270	14.881
0.625	16.227	15.892	16.266	16.128
0.315	22.846	22.020	20.798	21.885

Note: Con, concentration; Ct, Cycle Threshold; AV, average.

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