Original Article

Development and Application of Reverse Transcription Loop-mediated Isothermal Amplification (RT-LAMP) for Feline Coronavirus Detection

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Abstract

Mutated feline coronavirus (FCoV) causes a highly fatal disease in cats, named feline infectious peritonitis (FIP). Common FIP clinical signs represent an accumulation of fluid in the thorax and/or abdomen. Screening tests currently rely on fluid analysis with cytologic examination to differentiate cellular components. Accordingly, accurate ante-mortem diagnosis is prerequisite for effective treatment strategies. This study focused on developing a rapid diagnostic tool for FCoV by using reverse transcription loop-mediated isothermal amplification (RT-LAMP). Clinical samples composed of bodily fluids, plasma and feces were obtained from FIP-suspected cats (n = 63), apparent healthy cats living with (n = 12) and without (n = 10) clinically ill cats. Following RNA extraction and quantification, the modified RT-LAMP targeting the highly conserved 3'-untranslated region (3'UTR) was thoroughly performed and results were compared to reverse transcription polymerase chain reaction (RT-PCR). The analysis focused on sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). The results showed that all submitted feline fluid samples were 38% (24/63) positive by RT-PCR and 44% (28/63) positive by RT-LAMP. Cats living in the same household with FIP-suspected cats strikingly displayed 98% (11/12) positivity when detected from plasma and/or feces. For cats with no history of previous FIP exposure, the results demonstrated that 30% (3/10) tested positive by RT-PCR compared to 50% (5/10) by RT-LAMP. Additionally, RT-LAMP represented a diagnostic sensitivity and NPV at 100%, while specificity and PPV were over 80%. In conclusion, RT-LAMP assay is applicable to confirm FIP infection in clinical case and to monitor FCoV carrier in healthy cats.

Keywords: 3'UTR, feline coronavirus, feline infectious peritonitis, RT-LAMP, RT-PCR

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บทคัดย่อ

การพัฒนาและประยุกต์ใช้เทคนิค Reverse transcription loop-mediated isothermal amplification (RT-LAMP) ในการตรวจหาเชื้อโคโรนาไวรัสในแมว

สมพร เตชะงามสุวรรณ อารยา รัตนกถิกานนท์ รุ่งโรจน์ ธนาวงษ์นุเวช*

โรคเยื่อบุช่องท้องอักเสบในแมว (FIP) เกิดจากเชื้อโคโรนาไวรัสในแมว (FCoV) ที่มีการกลายพันธุ์ สัตว์ที่ป่วยจะมีการสะสมของของเหลวในช่องอก และ/หรือ ช่องท้อง การตรวจคัดกรองเบื้องต้นมักอาศัยการตรวจตัวอย่างของเหลวเพื่อจำแนกชนิดของเซลล์ นอกจากนี้การตรวจวินิจฉัยที่แน่นอนในขณะที่สัตว์ยังมีชีวิตอยู่นับว่ามีความสำคัญต่อการวางแผนการรักษา การศึกษาในครั้งนี้มีวัตถุประสงค์เพื่อพัฒนาวิธีการวินิจฉัยเชื้อ FCoV ในประเทศไทย ด้วยวิธีการ Reverse transcription loop-mediated isothermal amplification (RT-LAMP) ตัวอย่างทางคลินิกประกอบด้วยของเหลวจากช่องว่างภายในร่างกาย พลาสมา และอุจจาระของแมวที่สงสัยว่าป่วยเป็น FIP จำนวน 63 ตัว แมวปกติที่อยู่ร่วมกับแมวป่วยจำนวน 12 ตัว และแมวที่อยู่ตัวเดียวจำนวน 10 ตัว หลังจากการสกัดและวัดปริมาณสารพันธุกรรมจากตัวอย่างดังกล่าว นำมาทดสอบด้วยเทคนิค RT-LAMP โดยเปรียบเทียบกับเทคนิค RT-PCR ต่อจีนในตำแหน่งอนุรักษ์ 3'-untranslated region (3'UTR) ผลการวิเคราะห์พบแมวที่สงสัยว่าป่วยเป็น FIP ให้ผลบวกร้อยละ 38 และ 44 เมื่อตรวจด้วยเทคนิค RT-PCR และ RT-LAMP ตามลำดับ แมวปกติที่อยู่ร่วมกับแมวป่วยให้ผลบวกร้อยละ 98 ในทั้งสองวิธี และแมวที่อยู่ตัวเดียวให้ผลบวกร้อยละ 30 และ 50 ตามลำดับ เมื่อนำมาวิเคราะห์ค่าความไวของการทดสอบ (sensitivity) และค่าพยากรณ์ลบ (NPV) ของเทคนิค RT-LAMP พบว่าให้ผลร้อยละ 100 ในขณะที่ค่าความจำเพาะของการทดสอบ (specificity) และค่าพยากรณ์บวก (PPV) ให้ผลมากกว่าร้อยละ 80 ดังนั้นเทคนิค RT-LAMP จึงสามารถนำมาใช้ตรวจคัดกรองแมวป่วยและแมวที่เป็นพาหะได้

คำสำคัญ: 3'UTR ไวรัสโคโรนาในแมว เยื่อบุช่องท้องอักเสบติดต่อในแมว RT-LAMP RT-PCR

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Introduction

Loop-mediated isothermal amplification (LAMP) is a powerful innovative gene amplification tool developed for early detection and identification of microbial disease. It is a simple, rapid, specific and cost-effective nucleic acid amplification method. Amplification and detection of each specific gene can be completed in a single tube by incubating mixture of samples, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature (about 60-65^{0}C). The whole procedure can be completed in less than an hour. Because of its high specificity, the presence of amplified product indicates the presence of the target gene. The products can be visualized by naked eyes, agarose gel electrophoresis or with hydroxynaphthol blue dye addition (Parida et al., 2008; Cardoso et al., 2010).

Coronaviruses are enveloped, positive-sense RNA viruses belonging to the subfamily Coronavirinae in the family Coronaviridae. Coronaviruses cause varied ranges of diseases in broad host spectrum not only found in humans but also in wild and domesticated animals including severe acute respiratory syndrome (SARS) in humans, transmissible gastroenteritis (TGE) in pigs, infectious bronchitis virus (IBV) in chickens, canine coronavirus (CCoV) in dogs and feline coronavirus (FCoV) in cats. Although reverse transcription-LAMP assay is developed for the detection of several coronaviruses during the last decade (Pyrc et al., 2011; Ren and Li, 2011; Qiao et al., 2012), there has not been an attempt to use this technique on companion animal's coronaviruses.

Feline coronavirus (FcoV) consists of two biological types resulting in different forms of clinical manifestations. Infected cats with subclinical or mild diarrhea are usually caused by feline enteric coronavirus (FECV), while those with effusions (wet form) or caseous abscesses (dry form) in their bodily cavities are particularly mediated by feline infectious peritonitis virus (FIPV). Recently, incidence of FCoV isolates among cat populations in Thailand during 2010-2011 has been reported (Techangamsuwan et al., 2012). Cats with FIP clinically suspicions showed 46% positivity by RT-PCR targeting the highly conserved 3'-untranslated region (3'UTR). However, even the superior sensitivity and specificity of the PCR; it remains quite expensive for routine diagnosis. The objective of this study was to establish a simple, rapid diagnostic tool for feline infectious peritonitis disease

detection in Thailand using the reverse transcription loop-mediated isothermal amplification (RT-LAMP) compared to the RT-PCR.

Materials and Methods

Clinical specimens: All samples were collected at The Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University. Three categories of specimens comprised 70 thoracic and/or abdominal fluids collected from suspected FIP-infected cats (n = 63), 17 plasma and 21 feces collected from healthy cats living with (n = 12) and without (n = 10) FIP-suspected cats, respectively. Fluid, EDTA-anticoagulated whole blood as well as sterile phosphate buffer saline (PBS)-diluting feces were centrifuged at 3,000 rpm for 10 min. Supernatants and plasma were harvested and kept at -80^{0}C until used.

Nucleic acid extraction and RT-PCR assay: Viral RNA extraction using NucleoSpin® RNA virus (Macherey-Nagel, DÜren, Germany) following the manufacturer's instruction was done on 150 µl of each specimen. The concentration of RNA was quantified using NanoDrop spectrophotometer (Thermo Scientific, USA). Initially, the reverse transcription (RT) was performed using 500 ng RNA, random primers (Promega, Mannheim, Germany) and Omniscript RT-PCR kit (Qiagen, Hilden, Germany). The reaction was incubated at 60^{0}C for 1 hour using the thermoregulator ATC 401 (NYX Technik Inc., USA). Specific oligonucleotide primers for feline cDNA qualification (feline GAPDH) and FCoV detection (FCoV-3'UTR) were chosen from previous studies (Table 1) (Herrewegh et al., 1995; Penning et al., 2007).

Subsequently, the PCR reaction was carried out based on 2 µl cDNA, 0.4 µM (each) primers, 1.25 U Taq polymerase (Invitrogen), 1 mM MgSO4, and 0.2 mM (each) dNTPs with GoTaq Flexi buffer (Promega, Mannheim, Germany). The annealing temperatures were 50^{0}C (feline GAPDH) and 56^{0}C (FCoV-3'UTR). The PCR products were run on 1.5% agarose gels compared with the positive control (fresh tissue from a FIP necrosied cat diagnosed by histopathology) and the negative control (reaction omit the genomic RNA).

Positive amplicons were purified with Nucleospin® extract II kit (Macherey-Nagel, DÜren, Germany) and submitted for sequence analysis (1st BASE Pte Ltd, Singapore). Data were analyzed by Bioedit Sequence Alignment Editor version 7.0.5.3 (Hall, 1999) and compared with previously reported sequences available in GenBank.

LAMP primer designing and establishment of LAMP amplification: Based on results of sequencing analysis above, the conserved region of 3'UTR gene of FCoV (approximately 200 bp) was selected for the LAMP primer set designing by PrimerExplorer V4 software freely available at http://primerexplorer.jp/elamp4.0.0/index.html (Fig 1, Table 1).

The LAMP reaction was performed in 25 µl mixture containing 2 µl cDNA, 0.2 µM (each) outer primers (FCoV-F3 and FCoV-B3), 1.6 µM (each) inner primers (FCoV-FIP and FCoV-BIP), 8 U Bst DNA polymerase (New England Biolabs), 8 mM $MgSO_4$, and 0.8 M Betaine (Sigma Aldrich, 1.4 mM (each) dNTPs with ThermolPol buffer (New England Biolabs). Amplification was done at 60^{0}C for 2 hours and terminated at 80^{0}C for 2 min. The products were inspected on 2% agarose gels with a pattern of multiple bands of different molecular weights.

Data analysis: All data from the RT-LAMP assays were comparatively analyzed with the data from the RT-PCR assays considered as a gold standard. The evaluation included sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV).

Results

Identification the apparently healthy FCoV carriers by detection of 3'UTR gene: The detection of FCoV RNA in various kinds of specimens including effusion from FIP-suspected cats, plasma and/or feces from apparently healthy cats living with those sick and living alone were thoroughly performed by RT-PCR targeting the 3'UTR gene. The specific amplicon was detected at 223 bp on agarose gel electrophoresis (Fig 2, Table 2). In cats with bodily accumulating fluid and highly suspected of FIP disease, 38% (24/63) were tested positive, while the healthy cats that lived in the same household with FIP-suspected cats strikingly displayed 98% (11/12) positivity when detected from plasma and/or feces. For the cats that lived alone, implying no previous FIP exposure, 30% (3/10) were tested positive.

Table 1 Oligonucleotide primers used.

Primer name	Type	Sequence (5'→3')	Length (bp)
Feline GAPDH	Forward primer	AGTATGATTCCACCCACGGCA	21
	Reverse primer	GATCTCGCTCCTGGAAGATGGT	22
FCoV-3'UTR	Forward primer	GGCAACCCGATGTTTAAAACTGG	23
	Reverse primer	CACTAGATCCAGACGTTAGCTC	22
FCoV-F3	Forward outer	AACCCGATGTTTAAAACTGG	20
FCoV-B3	Reverse outer	CCATTGTTGGCTCGTCAT	18
FCoV-FIP	Forward inner	ACTACACGTGCTTACCATTCTGTACATTCCGGGGAATTACTGG	43
FCoV-BIP	Reverse inner	CAAGCAACCCTACTGCATATTAGGAAGCGGATCTTTAAACTTCTCT	46

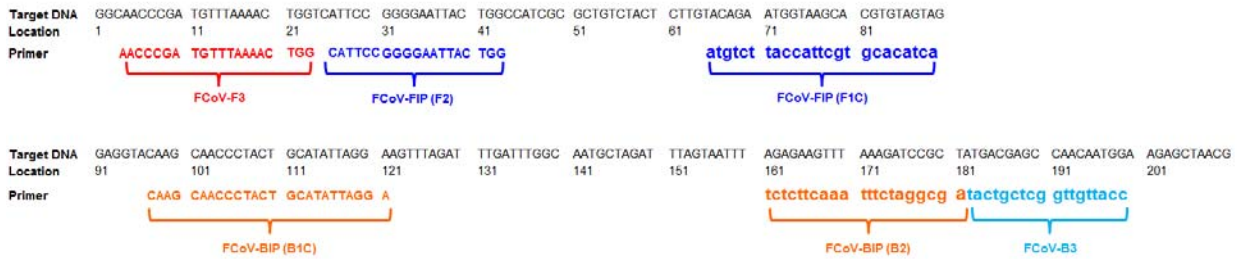

Figure 1 Different types of primers used in RT-LAMP reaction were designed based on the 210-bp conserved region of FCoV-3'UTR gene. Forward outer primer (FCoV-F3) located at nucleotide (nt.) 4th-23rd. Forward inner primer (FCoV-FIP) spanned 2 regions consisted of the F2 region (F2; nt. 25th-43rd) and the complement to the F1 region (F1C; nt. 88th-65th). Reverse/Backward inner primer (FCoV-BIP) spanned 2 regions consisted of the complement to the B1 region (B1C; nt. 97th-121st) and the B2 region (B2; nt. 181st-161st). Reverse outer primer (B3) located at nt. 199th-182nd

LAMP primer design: After verifying the sequencing results, the 210-bp selective conserved fragment of 3'UTR gene of FCoV was used for LAMP primer design via web-based interactive PrimerExplorer V4 software (Fig 1). The primers including outer primers (F3 and B3) and inner primers (FIP and BIP) are demonstrated in Table 1.

Detection of FCoV by a modified RT-LAMP: The modified RT-LAMP was performed in the same samples and directly compared with the conventional RT-PCR. After visualization by 2% agarose gel electrophoresis stained with ethidium bromide, the presence of a ladder-like pattern indicating different molecular weights products was considered a positive result (Fig 3, Table 2). By parallel comparison with the RT-PCR, the RT-LAMP increased the positivity up to 44% (28/63) and 50% (5/10) in suspected FIP cats and in apparently healthy cats, respectively. In addition, the RT-LAMP demonstrated its diagnostic sensitivity and negative predictive value (NPV) at 100%, while specificity and positive predictive value (PPV) were over 80% (*, Table 2).

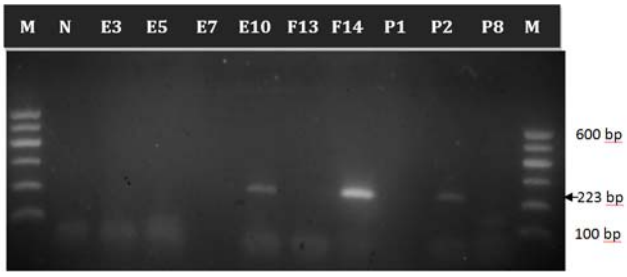

Figure 2 Analysis of FCoV by RT-PCR with 1.5% agarose gel electrophosis. M: 100 bp DNA ladder marker, N: negative, E: effusion, F: feces, P: plasma

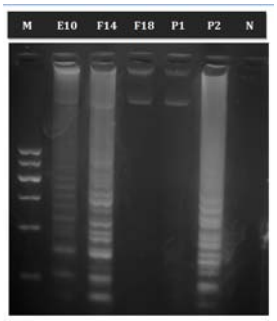

Figure 3 Analysis of FCoV by RT-LAMP with 2% agarose gel electrophosis. M: 100 bp DNA ladder marker, N: negative, E: effusion, F: feces, P: plasma

Table 2 Comparison between RT-PCR and RT-LAMP assays

		RT-PCR						
		Bodily fluids (63 FIP-suspected cats)		Plasma/Feces (12 cats living with FIP)		Plasma/Feces (10 cats living singly)		
		Positive	Negative	Positive	Negative	Positive	Negative	Total
RT-LAMP	Positive	24	4	11	0	3	2	44
	Negative	0	35	0	1	0	5	41
Total		24	39	11	1	3	7	85
Sensitivity[1]		100%		100%		100%		*100%**
Specificity[2]		89.7%		100%		71.4%		*87.2%**
PPV[3]		85.7%		100%		60%		*81.9%**
NPV[4]		100%		100%		100%		*100%**

[1] Sensitivity related to the RT-LAMP's ability to identify positive results when compared to the gold standard RT-PCR
[2] Specificity related to the RT-LAMP's ability to identify negative results when compared to the gold standard RT-PCR
[3] Positive predictive value (PPV) or precision rate is the proportion of subjects with positive test results correctly diagnosed.
[4] Negative predictive value (NPV) is the proportion of subjects with negative test results correctly diagnosed.
* Mean value of all groups

Discussion

One of the most life-threatening infectious diseases in cats is feline infectious peritonitis (FIP) disease caused by the mutated feline coronavirus (FCoV). The major obstacle to gain successful treatment is a lack of rapid and accurate ante-mortem diagnosis. Even though the distinguished symptom of FIP-suspected effusive form is an accumulation of pleural and/or abdominal fluids, several etiologies are needed to be differentially diagnosed including suppurative pleuritis/peritonitis, traumatic hemothorax/chylothorax, nephropathy and lymphoma-induced ascites (Sharif et al., 2010). Several laboratory aids have been implemented to overcome this burden, particularly molecular technique such as RT-PCR.

The RT-PCR has been developed for the detection of RNA genomic FCoV and for the differentiation of FCoV genotypes in clinical samples by emphasizing on the 3'UTR and S genes, respectively (Herrewegh et al., 1995; Lin et al., 2009; Techangamsuwan et al., 2012). However, the application of RT-PCR method is limited only to the best-equipped laboratory. To establish a simple rapid diagnostic tool for FIP disease, the reverse transcription loop-mediated isothermal amplification (RT-LAMP) is developed. We showed that RT-LAMP assay for FCoV provided better positivity rate (44-50%) with high sensitivity and specificity. This is in accordance with previous studies particularly in swine transmissible gastroenteritis coronavirus and turkey coronavirus showing the comparable detection limit, sensitivity and specificity of RT-LAMP assay with the gold standard PCR (Cardoso et al., 2010; Chen et al., 2010).

Although a better positivity rate was achieved, the method was not as simple as expected. From our experience, RT-LAMP assays still yielded a high rate of false positive incidence in the negative control group. In general, the LAMP assay is found to be 10-100 folds more sensitive than the routine PCR with a detection limit of 0.01-0.001 plaque forming unit (pfu) of virus (Parida et al., 2008). Accompanied by the set of 4 primers spanning 6 distinct sequences of target gene, only a minute amount of target sequence could be amplified. The detection of amplified products still required agarose gel electrophoresis due to the fact that the detection of turbidity by naked eyes was scarcely observed. Taken together, we suggested that the modified RT-LAMP assay for FCoV diagnosis could be used for the screening cases but could not be used for RT-PCR replacement.

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