Journal of Virological Methods 256 (2018) 32–36

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Detection of feline Coronavirus in effusions of cats with and without feline infectious peritonitis using loop-mediated isothermal amplification

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ARTICLE INFO

Keywords:
RT-LAMP
FIP
Diagnosis

ABSTRACT

Feline infectious peritonitis (FIP) is a fatal disease in cats worldwide. The aim of this study was to test two commercially available reaction mixtures in a reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay to detect feline Coronavirus (FCoV) in body cavity effusions of cats with and without FIP, in order to minimize the time from sampling to obtaining results.

RNA was extracted from body cavity effusion samples of 71 cats, including 34 samples from cats with a definitive diagnosis of FIP, and 37 samples of control cats with similar clinical signs but other confirmed diseases. Two reaction mixtures (Isothermal Mastermix, OptiGene Ltd.and PCRun™ Molecular Detection Mix, Biogal) were tested using the same primers, which were designed to bind to a conserved region of the FCoV membrane protein gene. Both assays were conducted under isothermal conditions (61 °C–62 °C). Using the Isothermal Mastermix of OptiGene Ltd., amplification times ranged from 4 and 39 min with a sensitivity of 35.3% and a specificity of 94.6% for the reported sample group. Using the PCRun™ Molecular Detection Mix of Biogal, amplification times ranged from 18 to 77 min with a sensitivity of 58.8% and a specificity of 97.3%.

Although the RT-LAMP assay is less sensitive than real time reverse transcription PCR (RT-PCR), it can be performed without the need of expensive equipment and with less hands-on time. Further modifications of primers might lead to a suitable in-house test and accelerate the diagnosis of FIP.

Feline coronavirus (FCoV), a member of the genus *Alphacoronavirus* of the subfamily *Coronavirinae*, family *Coronaviridae* within the order *Nidovirales* (de Groot et al., 2011), belongs to a group of enveloped, positive-sense RNA viruses that cause diseases in several species, such as severe acute respiratory syndrome (SARS) in humans or transmissible gastroenteritis (TGE) in pigs. Despite the high prevalence of FCoV infections in the cat population worldwide, only 5–10% of FCoV-infected cats develop the fatal disease feline infectious peritonitis (FIP) (Addie and Jarrett, 1992). This change of virulence of a harmless FCoV biotype that usually causes no clinical signs into the pathogenic variant is thought to be caused by mutations in the FCoV spike protein gene (Chang et al., 2012; Vennema et al., 1998). These mutations cause a change in tropism from enterocytes to macrophages, giving FCoV the ability to infect and effectively replicate within cells of the macrophage lineage and cause a lethal systemic disease with multi-organ involvement (Pedersen, 2009). The median survival time of cats with effusive FIP is only a few days (Ritz et al., 2007), and the diagnosis of FIP commonly leads to euthanasia, since to date, no treatment has been proven to be effective. Cats with FIP show nonspecific clinical signs such as fever, weight loss and anorexia, often accompanied by body cavity effusions and/or ocular and neurological signs. A definitive diagnosis of FIP *ante mortem* remains challenging, especially when no body cavity effusions can be detected (Hartmann et al., 2003). Presently, the gold standard for the diagnosis of FIP is considered to be immunostaining of FCoV antigen in macrophages within tissue lesions, a technique that requires invasive tissue collection (Kipar and Meli, 2014). In cats with FIP, FCoV can be detected by RT-PCR in cell-free body cavity effusions in more than 80% of the cases, while serum or blood samples often are negative (Doenges et al., 2017). For both immunostaining and RT-PCR, samples have to be sent to specialized laboratories, resulting in the delay of diagnostic results. This leads to further unnecessary testing for other diseases, to withholding necessary therapy of other treatable diseases, or to delayed euthanasia in cats suffering from severe signs of FIP. Therefore, a fast and simple point of care test would be very beneficial in the diagnostic process.

Loop-mediated isothermal amplification (LAMP) is a simple, rapid, and cost-effective nucleic acid amplification method (Notomi et al., 2000) and is already used for the detection of Coronaviruses in humans

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https://doi.org/10.1016/j.jviromet.2018.03.003
Received 12 September 2017; Received in revised form 9 March 2018; Accepted 10 March 2018
Available online 11 March 2018

and several animal species (Hong et al., 2004; Nemoto et al., 2015). A set of four to six primers is used, that form products with self-hybridizing loop structures. By using a DNA polymerase with strand displacement activity, no melting or annealing steps are required, and amplification products of different lengths are formed at a constant temperature of 60–65 °C (Nagamine et al., 2002). Since LAMP reactions only require a simple heat block with constant temperature, and DNA amplification can be detected by fluorescence or color change, the method can be applied for point-of-care diagnostics (Surabattula et al., 2013).

The aim of this study was to test specificity and sensitivity of two commercially available reaction mixtures in a reverse transcription LAMP (RT-LAMP) to detect FCoV in body cavity effusions of cats with and without FIP, and to minimize the time from sampling to obtaining results.

This study included 71 cats that were presented to the Clinic of Small Animal Internal Medicine, LMU Munich, Germany. All cats included had body cavity effusions. In every cat presenting with body cavity effusions, FIP is a potential differential diagnosis. An earlier study showed that FIP is responsible for about 40% of effusions, while most of the remaining cases were caused by malignomas, cardiac insufficiency or purulent serositis (Hirschberger et al., 1995). The FIP group (n = 34) included cats with a definitive diagnosis of FIP by one or more methods: All effusions of cats with FIP tested positive for FCoV by RT-PCR by a commercial laboratory, and in 26/34 samples putative disease-causing mutations could be detected. The RT-PCR detection method has been described previously (Felten et al., 2017). In 25/34 cats FIP diagnosis was achieved by post-mortem examination, including full body necropsy with histopathological examination. Diagnosis of FIP was confirmed when typical histologic lesions where detected (surface-bound multi-systemic pyogranulomatous and fibrinonecrotic disease with venulitis with or without high-protein exudate). In 17/25 cats with full body necropsy immunohistological staining for FCoV-antibody was done on tissue sections and returned a positive result. Immunofluorescent staining of FCoV antigen in macrophages of thoracic or abdominal effusion was done in 20/34 cats, and all samples returned a positive result. A summary of the cases in the FIP group can be found in the supplementary Table 1.

Cats were included in the control group (n = 37) if they were definitively diagnosed with a disease other than FIP that explained the effusion. Cats of the control group suffered from neoplasia (n = 20), decompensated cardiac diseases (n = 12), inflammatory diseases (n = 2), such as bacterial peritonitis and pleurisy, or other diseases (n = 3). One cat had chronic thoracic chylous effusion of unknown origin and secondary fibroplastic pleurisy. In another cat, an end stage kidney disease caused effusion, and one cat had thoracic effusion after subcutaneous urethral bypass placement, which resolved after treatment. The diseases of the cats of the control group (n = 37) were definitively confirmed ante-mortem (n = 18) or at necropsy with histopathological examination (n = 19). Ante-mortem diagnosis was established by echocardiography for cardiac diseases (n = 8), and by cytology for neoplasia (n = 10). Immunofluorescent staining of FCoV antigen in macrophages of thoracic or abdominal effusion was done in 11/37 cats, with three positive and eight negative results. All effusions of the cats in the control group were tested for FCoV by RT-PCR, and all results were negative. The RT-PCR detection method has been described previously (Felten et al., 2017). A summary of the cases in the control group can be found in supplementary Table 2.

Body cavity effusion samples of all cats were obtained *ante mortem* with ultrasound guidance for diagnostic purposes. The use of samples for this study was approved by the Institutional Animal Care and Use Committee ('Ethikkommission des Zentrums für klinische Tiermedizin'), permission number 32-25-06-2014. Samples were stored at -80 °C in a 1.5 ml Eppendorf Safe-Lock microcentrifuge tube until assayed. All samples were centrifuged for 20 s at 15,000 × *g*. The supernatant of centrifuged thoracic and abdominal fluids was used for RNA extraction. When using fresh fluid samples, omission of the centrifugation step should be considered to include intact cells with a high viral burden (Pedersen et al., 2015). In thawed samples, cell integrity is lost and cell debris can be removed. Viral RNA was isolated using the commercial ZR Viral RNA KIT™ (Zymo Research Corp.) following the manufacturer's instructions. Briefly, 100 µl aliquots of samples were mixed with a buffer that facilitates viral particle lysis and allows for RNA adsorption onto the matrix of the Zymo-Spin™ Column. Then the RNA was washed and eluted with 15 µl of RNase free water. Extracted RNA aliquots were stored at −80 °C in an Eppendorf 1.5 ml Safe-Lock microcentrifuge tube until further processing.

The RT-LAMP primer design was assisted by the software PrimerExplorer (https://primerexplorer.jp/e/). Based on sequence analysis, the gene for the membrane protein (M) was selected as a target because it is highly conserved among FCoV strains. The DNA sequence from position 26,500 to 27,000 of the FCoV strain Black (GenBank accession number: EU186072.1) was used to design the RT-LAMP primers used in this study. A set of six primers, including two outer primers (forward primer F3 and backward primer B3), two inner primers (forward inner primer FIP and backward inner primer BIP), and two loop primers (forward loop primer LoopF and backward loop primer LoopB) were selected as the target sequence (Fig. 1 and Table 1).

Detection of FCoV was performed using RT-LAMP. Two different commercial reaction mixtures (Isothermal Mastermix by OptiGene Ltd., UK, PCRun™ molecular detection mix by Biogal, Israel,) were compared using the same set of primers.

For the amplification following the Isothermal Mastermix protocol, the total volume of 25 µl per reaction tube included 15 µl Isothermal Master Mix, 5 µl template, 5 µl Primer Mix and 0.1 µl SuperScript® III Reverse Transcriptase (Thermo Scientific). The Primer Mix consisted of 5 pmol each of F3 and B3 primers, 20 pmol each of FIP and BIP primers and 10 pmol each of LoopF and LoopB primers. For negative control, 5 µl water were added instead of 5 µl template. The reaction mix was incubated at 62 °C for 75 min in a 7500 Real-Time PCR System (Applied

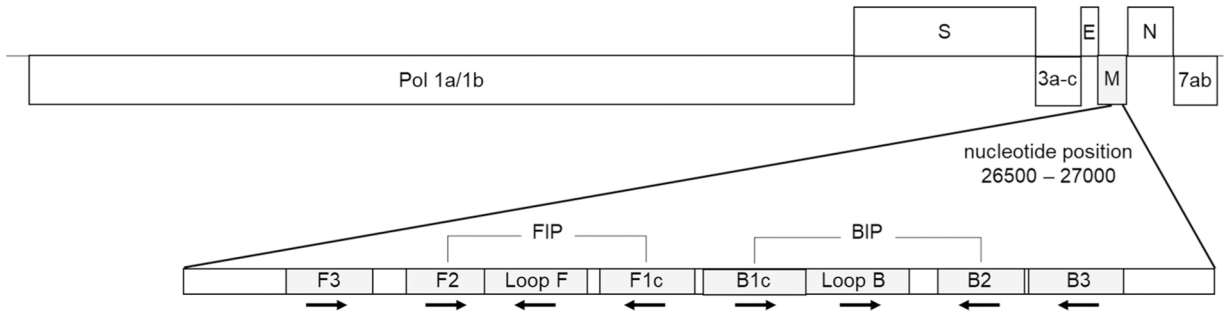

Fig. 1. Position and orientation of RT-LAMP primers. The upper part shows the genomic organization of the FCoV genome. In the lower part the positions of the oligonucleotides used as LAMP-primers in the gene of the membrane protein (M) are shown. Pol 1a/1b,: Polymerase 1a and 1b gene; S, spike protein gene; 3a-c, gene cluster 3abc; E, envelope protein gene; N, nucleocapsid protein gene; 7ab, gene cluster 7ab.