



## NOTE

Virology

# Detection of feline norovirus using commercial real-time RT-PCR kit for the diagnosis of human norovirus infection

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83(5): 805–808, 2021

doi: 10.1292/jvms.20-0703

Received: 10 December 2020

Accepted: 2 March 2021

Advanced Epub:

11 March 2021

**ABSTRACT.** Feline noroviruses (FNoVs) are potential clinical pathogens in cats. To perform an epidemiological study of FNoV infection, it is necessary to develop a simple and effective method for virus detection. We investigated whether a commercial human NoV quantitative RT-PCR kit for the detection of human NoVs used in medical practice can be applied for FNoV detection. This kit was capable of detecting the FNoV gene regardless of the genogroup (GIV and GVI) in experimental and field samples. Based on the above findings, it is possible to detect FNoVs using human NoV tests. The relationship between FNoV infection and gastroenteritis in cats may be clarified by applying these methods to an epidemiological survey of FNoVs.

**KEY WORDS:** commercial human kit, diagnostic method, feline norovirus (FNoV), genogroup

Noroviruses (genus *Norovirus*, family *Caliciviridae*) are non-enveloped viruses with positive-sense single strand RNA genomes of approximately 7.5 kb coding three open reading frames (ORFs) [2]. ORF1 encodes NTPase (helicase), Vpg, and RNA-dependent RNA polymerase (RdRp), ORF2 encodes the major capsid protein VP1, and ORF3 encodes the minor capsid protein VP2 [6]. Noroviruses (NoVs) are divided into seven genogroups, GI–GVII [18]. These genogroups are further classified into genotypes (e.g. GIV NoVs are classified into genotypes 1–3, GIV.1–GIV.3). NoVs of carnivores, including dogs and cats, are divided into 3 different genogroups (GIV: GIV.2, GVI: GVI.1, and GVI.2, GVII) [4]. Phylogenetic analysis of GIV and GVI carnivore NoVs found them to be closely related to GII and GIV human NoVs [3].

Feline norovirus (FNoV) is detected in fecal samples from animals with gastroenteritis [3, 13, 15]. We previously reported that gastroenteric signs were noted in cats orally inoculated with GVI.1 FNoV [16, 17]. Therefore, FNoVs are potential clinical pathogens in cats. However, epidemiological information on these viruses are limited [15]. One of the reasons for this is the absence of an effective method to detect FNoVs. At present, FNoVs are detected using universal *Caliciviridae* primers (P289d/P290d), but this method has 3 disadvantages. First, it detects both FNoVs and vesivirus (feline calicivirus: FCV), belonging to *Caliciviridae* [11, 15]. Second, the primers used have been suggested to detect rotavirus genes [8]. Third, genes other than viral genes are likely to be nonspecifically detected. To perform an epidemiological study of FNoV infection in cats, it is necessary to develop a simple and effective methods for virus detection. We investigated whether quantitative RT-PCR (qRT-PCR) used to detect human NoVs in medical practice can be applied for FNoV detection.

We investigated whether the primer pairs generally used to detect the human NoV gene are capable of detecting the FNoV gene. Fifteen of GIV FNoV-positive fecal samples were collected from 6-month-old two cats experimentally inoculated with GIV.2 FNoV M81 strain (GenBank accession No. LC389583). Fifteen of GVI FNoV-positive fecal samples were collected from GVI.2 FNoV M49-1 strain-infected cats as previously described [16, 17]. For the norovirus-negative control, fifteen of fecal samples from specific pathogen-free (SPF) cats and five of FCV-positive field fecal samples were used. Animal experiments were approved by the President of Kitasato University through the Institutional Animal Care and Use Committee of Kitasato University (17-032), and performed in accordance with the Guidelines for Animal Experiments of Kitasato University. For the NoV-positive control, five of GII human NoV-positive fecal samples and two of GI human NoV-positive fecal samples were used (These samples and some samples from cats were kindly provided by Health and Environment Research Institute, Yokkaichi, Japan). For the purified FCV, the FCV F4 strain stored at our laboratory was used. All fecal samples were diluted 1:10 in phosphate-buffered saline (PBS), and centrifuged at 1,000 g for 15 min at 4°C. Total RNA was isolated from fecal samples using the High Pure Viral RNA Isolation Kit (Roche Diagnostics, Indianapolis, IN, USA) following the manufacturer's instructions. Total RNA isolated from fecal samples was

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reverse transcribed with ReveTra Ace Reverse Transcriptase (TOYOBO, Osaka, Japan), generating first strand cDNA following the manufacturer's instructions. PCR amplification of the ORF1-ORF2 fragment was performed with the specific primers (COG1F/COG1R and COG2F+ALPF/COG2R) by the method described before [11] (Table 1). For all NoV- and FCV-positive samples, a specific band was detected by conventional RT-PCR using P289d/P290d primer set (Table 1, Data not shown). On the other hand, only GI human NoV was detected using COG1F/COG1R (Fig. 1A). Using COG2F+ALPF/COG2R, GIV and GVI FNoV were detected in addition to GII human NoV, but FCV was not detected (Fig. 1B). The GI human NoV detection rate using COG1F/COG1R was 100% (n=2), whereas those of the other viruses were 0%. The detection rate of each virus using COG2F+ALPF/COG2R was: 0% (GI human NoV; 0/2), 100% (GII human NoV; 5/5), 100% (GIV FNoV; 15/15), 93.3% (GVI FNoV; 14/15), and 0% (FCV; 0/5).

Fecal samples collected from 2 GIV FNoV-infected cats (n=30) and 8 GVI FNoV-infected cats (n=30) were randomly selected, and the viral load was measured using qRT-PCR. The correlation between the values of viral load assessed using GIV FNoV-specific or GVI FNoV-specific qRT-PCR (FNoV qRT-PCR) and commercial human norovirus detective qRT-PCR (human NoV qRT-PCR) was investigated. FNoV qRT-PCR with the specific primers and probe (Table 1) was performed as described previously [16]. The RNA copy number was calculated following the procedure described by Fronhoffs *et al.* [5]. Detection of FNoV RNA using a commercial human norovirus detection kit (TaKaRa® qPCR Norovirus (GI/GII) Typing Kit; TaKaRa Bio, Kusatsu, Japan) was carried out according to the manufacturer's protocol. Based on the results of conventional RT-PCR, the primers and probe set to detect the GII human NoV gene were used in human NoV qRT-PCR. A correlation was noted between the viral loads determined using feline NoV qRT-PCR and human NoV qRT-PCR in the samples from GIV FNoV-infected cats (Fig. 1C,  $r^2=0.9598$ ). The samples from GVI FNoV-infected cats produced similar results (Fig. 1D,  $r^2=0.9756$ ).

A total of 100 fresh fecal samples in the litter boxes and on the ground in the animal shelter were collected from cats between 2016 and 2018 in Mie Prefecture were tested by universal *Caliciviridae* RT-PCR using P289d/P290d primer set and the human NoV qRT-PCR (Table 2). Thirteen samples were positive by universal *Caliciviridae* RT-PCR, whereas four samples were positive by the human NoV qRT-PCR. All human NoV qRT-PCR-positive samples were positive on universal *Caliciviridae* RT-PCR. When the PCR products were subjected to sequencing analysis as described previously [17], all samples positive on both universal *Caliciviridae* RT-PCR and human NoV qRT-PCR were FNoV, whereas all samples positive on only universal *Caliciviridae* RT-PCR were FCV. When the genogroups of 4 FNoV-positive samples were investigated using feline NoV qRT-PCR, one sample was GIV FNoV and all others were GVI FNoV.

FNoV infection in cats has been confirmed in the USA, Italy, and Japan [3, 13, 15]. FNoV is considered to be the cause of gastroenteritis in cats, but no clear evidence has been reported. As a reason for that there are no established methods for FNoV detection. In this study, we demonstrated that the commercial human NoV detective kit is capable of detecting FNoV.

When the primers (COG2F+ALPF/COG2R) and probe set detecting the GII human NoV gene were used for conventional RT-PCR, all FNoVs were detected regardless of the genogroup. The ALPF primer was prepared based on the gene sequence of the GIV human NoV Alphanon strain [11]. For that reason, GIV FNoV and GVI FNoV, which are phylogenetically close to the NoV Alphanon strain, can be detected using the (COG2F+) ALPF-COG2R primer set.

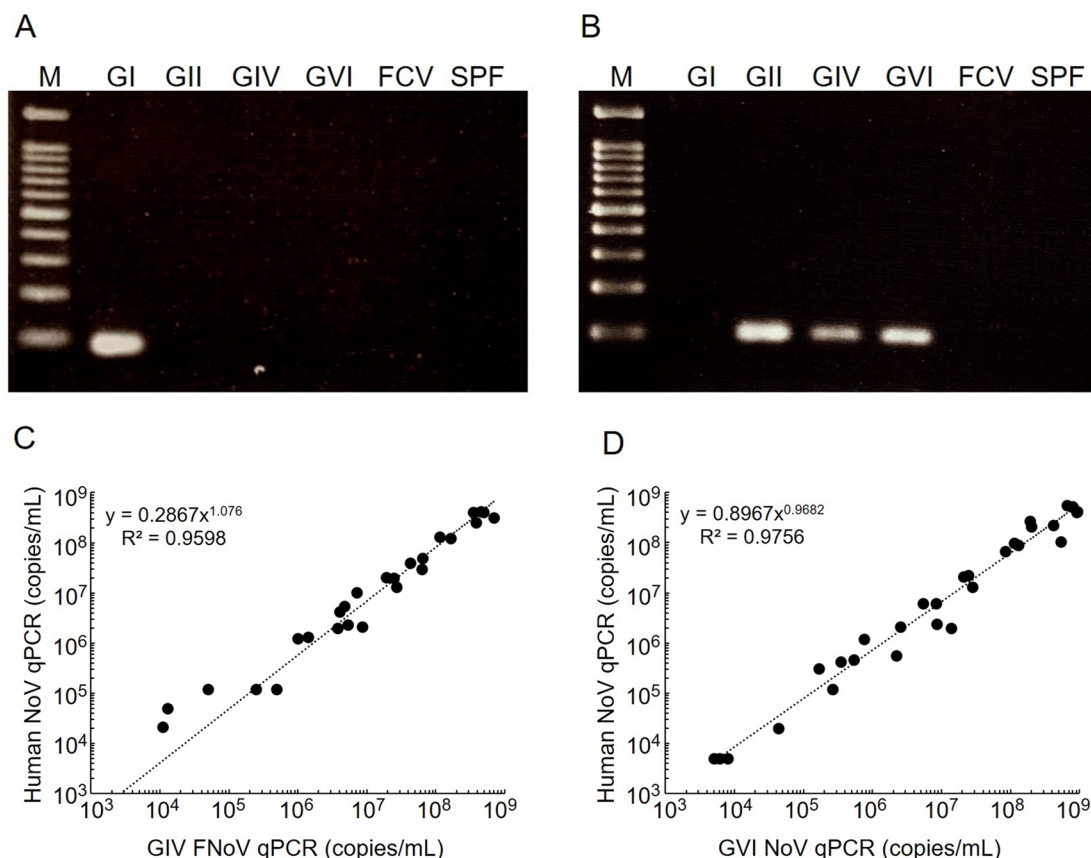
Even though the human NoV qRT-PCR is applied to detect the HNoV, we were able to detect the FNoV using this kit. It was unclear why the human NoV qRT-PCR was able to detect the FNoV. If ALPF is used as the primer in this kit, FNoV will be detected as with conventional PCR. However, the sequences of primers and probe included in the human NoV qRT-PCR have not been published. We confirmed that FNoV was detected by qRT-PCR reported by Obara *et al.* [12] in our preliminary experiment. In this previous report, ALPF is used as a primer. We speculate that the human NoV qRT-PCR contains ALPF and FNoV have been detected.

The FCVs are detected in feline feces [10]. The conventional method to detect FNoV is universal *Caliciviridae* RT-PCR. It is difficult to distinguish FCV and FNoV using this method unless the gene sequencing of the PCR products. FCV (331 bp) and

**Table 1.** Primer sequences used in this study

Name	Nucleotide sequence	Use	Reference
COG1F	5'-CGYTGGATGCGNTTYCATGA-3'	Conventional RT-PCR	[7]
COG1R	5'-CTTAGACGCCATCATCATTYAC-3'		
COG2F	5'-CARGARBCNATGTTYAGRTGGATGAG-3'	Conventional RT-PCR	[7, 11]
COG2R	5'-TCGACGCCATCTTCATTCACA-3'		
ALPF	5'-TTTGAGTCCATGTACAAGTGGATGCG-3'		
GIV VP1-sense	5'-CCTTCCTGGTTCCGCCAAC-3'	GIV feline norovirus (FNoV) quantitative RT-PCR (qRT-PCR)	This study
GIV VP1-antisense	5'-GCCACCTGGAGTTGGACATATC-3'		
GIV VP1-probe	5'-FAM-TCGTTCTCAGTCCCTGGCTTGGTCGTC-BHQ1-3'		
GVI VP1-sense	5'-GCAACTTGAGCCCCATAGTCTTG-3'	GVI FNoV qRT-PCR	[17]
GVI VP1-antisense	5'-CCAGAGTTCGCACGCAGG-3'		
GVI VP1-probe	5'-FAM-AAGGCACAACCTGTCCACTTCAACCAGC-BHQ1-3'		

For human norovirus (NoV) qRT-PCR, the primers and probe set included in the commercial kit were used.



**Fig. 1.** Detection of feline norovirus (FNoV) by conventional and quantitative RT-PCR (qRT-PCR) using human norovirus (NoV)-specific primers. A and B, Detection of human and feline NoV from fecal samples by conventional RT-PCR using human NoV-specific primers. A. Conventional RT-PCR results based on GI human NoV-specific primers (COG1F-COG1R). B. Conventional RT-PCR results based on GII human NoV specific primers (COG2F/ALPF-COG2R). C and D, Correlation of fecal FNoV viral RNA loads between FNoV qRT-PCR and human NoV qRT-PCR. C. Correlation of GIV FNoV RNA loads measured by GIV FNoV and human NoV qRT-PCR. D. Correlation of GVI FNoV RNA loads measured by GVI FNoV and human NoV qRT-PCR.

**Table 2.** The detection of feline norovirus (FNoV) and feline calicivirus (FCV) in field fecal samples from cats by universal *Caliciviridae* RT-PCR, and human norovirus (NoV) quantitative RT-PCR (qRT-PCR)

		Universal <i>Caliciviridae</i> RT-PCR		
		Positive (n=13)		Negative (n=87)
		FNoV (n=4)	FCV (n=9)	
Human NoV qRT-PCR	Positive	4	0	0
	Negative	0	9	87

FNoV (319 bp) can be distinguished based on the band position after electrophoresis on 6.5% polyacrylamide gel, but this is difficult for persons other than skilled researchers. Moreover, FCV and FNoV may be mixed in a singlet band on agarose gel. Therefore, the universal *Caliciviridae* RT-PCR may be inappropriate for an epidemiological survey of FNoV. Using the commercial human NoV qRT-PCR, FNoV was able to specifically detect. As the results of two tests were in correspondence, the commercial human NoV qRT-PCR is appropriate for an epidemiological survey of FNoV.

Diarrhea is a common clinical sign in cats. There are many causative agents, but a number of cases are diagnosed with an unknown cause. Moreover, in clinical veterinary practice, the only viruses tested as a cause of diarrhea are feline coronavirus and feline panleukopenia virus [1]. On the other hand, FNoV has been reported to be related to diarrhea in cats, but this virus is not included as a target cause because there is no clear evidence of a correlation between diarrhea and FNoV infection in cats. Using methods for FNoV detection, such as that used in this study, the relationship between FNoV infection and diarrhea may be clarified. Furthermore, it is necessary to investigate whether canine NoV (CNoV), which has a genome sequence similar to FNoV, can be detected by human NoV qRT-PCR. CNoV is associated with viral gastroenteritis in dogs [4, 9]. This kit will be useful for

epidemiological studies of norovirus infections in dogs.

We investigated whether qRT-PCR used to detect human NoVs in medical practice can be applied for FNoV detection [14]. Based on this finding, it is possible to detect FNoVs using human NoV tests. By applying this method to an epidemiological survey of FNoV, the relationship between FNoV infection and diarrhea may be clarified. FNoV researchers may be able to gather useful data using the commercial kit for virus detection.

CONFLICT OF INTEREST. The authors declare no conflict of interest.

ACKNOWLEDGMENT. This work was in part supported by JSPS KAKENHI Grant Number 19K06407.

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