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Original Article

Isolation and Molecular Detection of Feline Infectious Peritonitis Virus

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Abstract

Feline coronavirus (FCoV) is an enveloped single-stranded RNA virus, affecting wild and domestic cats. Feline infectious peritonitis viruses (FIPV) variants of FCoV cause fatal peritonitis affecting approximately 5% of FCoV infected animals. The present study aimed to detect and isolate the feline infectious peritonitis virus for the first time in Iraq. In this study, 50 samples (fecal swab and peritoneal fluid) were collected from suspected pet cats from different areas of Baghdad, Iraq. The very suitable age was under two years old. Real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) was used to detect Feline infectious peritonitis in infected collected samples by the amplification of spike protein (S). The result of real-time RT-PCR revealed that out of 50 samples from suspected cats, 10 samples were positive for FIPV. Moreover, 10 positive samples by real-time RT-PCR were used for the isolation of the virus in chicken embryo fibroblast cell culture. Subsequently, the isolated virus was detected by real-time RT-PCR and then by conventional RT-PCR, followed by electrophoresis.

Keywords: Cell culture, Feline coronavirus, Feline infectious peritonitis virus, Real-time RT-PCR

1. Introduction

Feline coronavirus (FCOV) is an enveloped single-stranded RNA virus that infects wild and domestic cats (1). It has a large, capped, polyadenylated RNA genome of about 29 kb, belonging to the family Coronaviridae within the order Nidovirales, and the subfamily *Coronavirinae*, genus *Alphacoronavirus*, and species *Alphacoronavirus1* (2, 3) which consists of 11 open reading frames (ORFs). Two major ORFs encode a replicase, four ORFs encode the structural proteins S (spike), E (envelope), M (membrane), as well as N (nucleocapsid), and five ORFs encode the nonstructural proteins 3a, 3b, 3c, 7a, and 7b (4). The FCOV most commonly causes asymptomatic infection or mild diarrhea but is also associated with a lethal systemic disease and feline infectious peritonitis. Consequently, feline coronaviruses are often divided into two groups: the highly pathogenic strains-feline infectious peritonitis viruses (FIPV), and the less severe feline enteric coronaviruses (FECV) (5).

The FIPV variants of FCOV cause fatal peritonitis, affecting approximately 5% of FCOV-infected animals (6). Cats with a poor cell-mediated immune response develop the acute, effusive or "wet" form of the disease, which is an immune complex vasculitis. This causes leakage of fluid from the blood vessels into the abdominal and chest cavities, leading to a distended abdomen, difficulty breathing, and the compression of the lungs. In cats with partial cell-mediated immunity, the chronic, non-effusive, or "dry" form develops, with pyogranulomatous or granulomatous lesions occurring in multiple tissues and organs (7).

Dry FIP may become effusive in the terminal stages of the disease when the immune system collapses. The most obvious clinical sign of an effusive form of the disease is the accumulation of fluid within the abdomen or chest with associated breathing difficulties (8). Other symptoms include lack of appetite, fever, weight loss, jaundice, and diarrhea. In the non-effusive form, lesions commonly occur in the eyes and central nervous system which may cause seizures or paralysis (9). Granulomas may also be observed in the peritoneal cavity, leading to more diverse and often vaguer clinical signs. The less severe infection may lead to a runny nose, discharge from the eyes, or mild diarrhea (7). In light of the aforementioned issues, the present study aimed to detect feline infection peritonitis by traditional and molecular techniques for the first time in Iraq.

2. Materials and Methods

2.1. Sample Collection, RNA Extraction, and cDNA Synthesis

A total of 50 samples (fecal swab and peritoneal fluid) were collected from pet cats between October 2020 and April 2021**.** The RNA extraction was performed with High pure Viral Nucleic acid and a purification kit (Kylt® RNA/DNA purification/German) commercial kit for obtaining complementary DNA (cDNA) from total RNA. Thereafter, a real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed accordingly. The samples were tested by RT-PCR according to the Vet qPCR real-time™ FCoV (Bioingentech/Chile), followed by cell culture. The positive result from RT-PCR was taken up for cell culture propagation of the virus. Subsequently, oligonucleotide primers were used, and the sequences are listed in table 1.

Table 1. Oligonucleotide sequences primers with probes for detection of Feline infectious peritonitis virus

Primer	Sequences (5'---3')
Forward primer	5' GGCAACCCGATGTTTAAAACTGG 3'
Reverse primer	5'CACTAGATCCAGACGTTAGCTC3'

Finally, an Electron microscope was used to detect the morphology of viruses.

2.2. Reverse Transcription

The reverse transcription process was performed according to the manufacturer protocol as follows:

- After thawing all frozen reagents, a briefly centrifuged, 2 µl of each RNA sample was mixed with 4.5 µl of cDNA synthesis premixture in a separate nuclease-free PCR tube, and the volume was adjusted to 10.5 µl by the addition of 4µl of PCR grade water.
- The mixture tube was placed in the thermal block for 1 cycle of the initial denaturation step at 60°C for 10 min; thereafter, the reaction was stopped by chilling the mixture at 4°C till the next step.
- The next step involved the addition of 1µl of Lig™RNAse inhibitor solution and Bio™ Transcriptase solution each to the mixture tube and raising the volume to 13.5µl.
- The reaction mix tube of each sample was loaded into a thermocycler preprogrammed at 1 cycle of annealing step at 25°C for 10 min, 1 cycle of extension step at 37°C for 60 min, and a final cycle of denaturation at 70°C for 10 min, producing cDNA for the later amplification reaction.

2.3. Amplification Of Nucleic Acid by Real-Time Reverse transcription-polymerase Chain Reaction

According to the manufacturer protocol, the amplification of cDNAs by real-time RT-PCR was completed by preparing the master mix of 5.5 µl VetqPCR- real-time FCoV for each sample, mixed with 2 µl of cDNA of the sample or negative/positive controls. The final reaction volume was adjusted to 13.5 µl by the addition of 6 µl of PCR grade water. The amplification of cDNA in a real-time thermocycler was performed as the program presented in table 2.

Table 2. Amplification of cDNAs by the real-time reverse transcription-polymerase chain reaction

		Temp.	Time
X 1 cycles	Initial Denaturation	94°c	2 min.
X 45 cycles	Denaturation	95°c	15 second
	Annealing	60°c	60 second

2.4. Virus Isolation

Virus isolation was performed by cultivation on chicken embryo fibroblast cell culture. The cytopathic effects (CPE) were observed after 24 hours post-inoculation. When CPE appeared under an inverted microscope, the picture was taken for investigation and the infected fluid was stored at -70°C until use for virus detection. Following that, real-time RT-PCR was used to detect the isolated virus on cell culture.

3. Results

3.1. Real-Time Reverse Transcription-Polymerase Chain Reaction from Collected Samples

Out of 50 samples, 10 samples illustrated positive results for the FIP virus by real-time RT-PCR as displayed in figure 1.

3.2. Virus Inoculation on Cell Culture of Chicken Embryo Fibroblast

After sample inoculation into chicken embryo fibroblast cell culture, CPE was noticed after 24 h. Thereafter, CPE was completed after 48 h. The virus was propagated on cell culture for three passages. The CPE was noticed on three passages on cell culture (Figures 2-5).

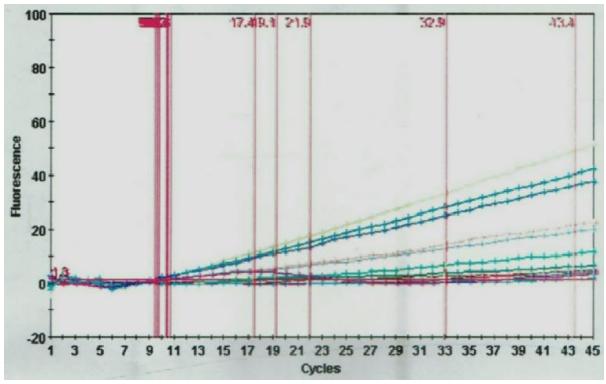

Figure 1. Amplification of real-time polymerase chain reaction for the detection of infectious peritonitis viruses showed CT value of positive samples with amplification of s gene

Figure 2. Control, uninfected chicken embryo fibroblast monolayer (100x)

Figure 3. Cytopathic effect of feline infectious peritonitis on chicken embryo fibroblast cell culture (first passage), illustrating the rounding of infected cells and syncytia formation (100x)

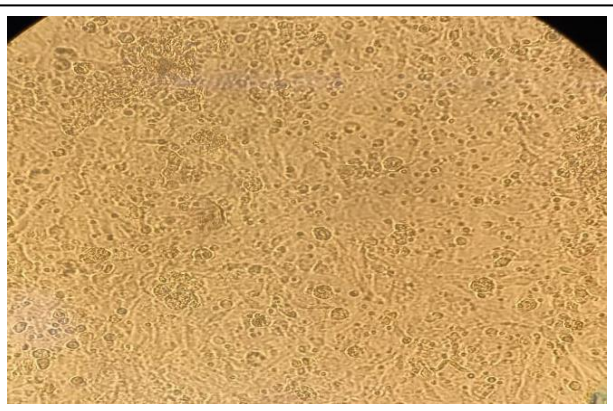

Figure 4. Cytopathic effect of Feline infectious peritonitis virus on chick embryo fibroblasts cell culture, displaying the rounding of infected cells (passage second) and syncytia formation after 24 hours post-inoculation (100x)

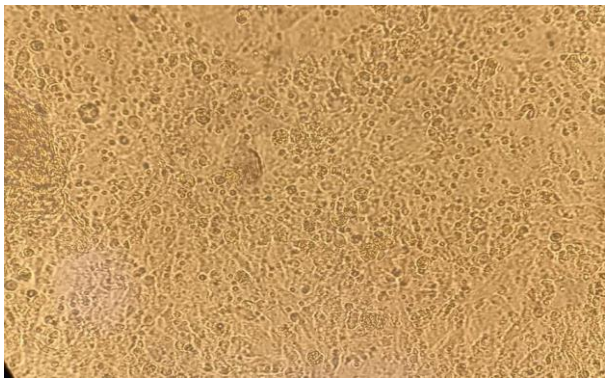

Figure 5. Cytopathic effect of Feline infectious peritonitis on chick embryo fibroblasts (CEF) cell culture after 24 hours (passage three), demonstrating the rounding of infected cells and syncytia formation after 24 hours post-inoculation

3.3. Real-Time Polymerase Chain Reaction after Cultivation on Cell Culture

Real-time RT-PCR was positive to detect the FIP virus on chicken embryo fibroblast cell culture as depicted in figure 6.

3.4. Result of Conventional Polymerase Chain Reaction for the Detection of Feline Infectious Peritonitis Virus Using Primer

Gel electrophoresis for amplified samples with primer revealed the presence of bands with 223 bp of amplified gene figure 7. Electron Microscope was used to detect FIP virus morphology (Figure 8), as previously described by Herrewegh (10).

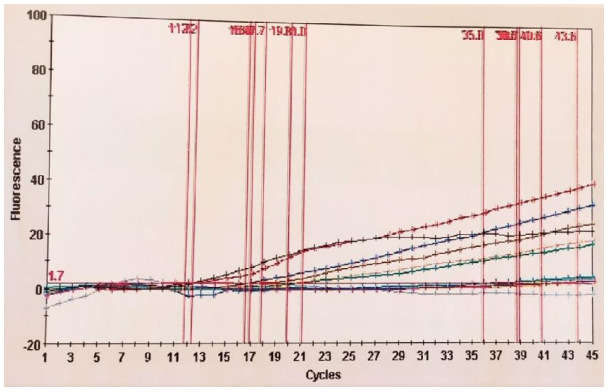

Figure 6. Plot of amplification of real-time reverse transcription-polymerase chain reaction for the detection of isolated Feline infectious peritonitis virus on cell culture showed positive samples with amplification of S gene

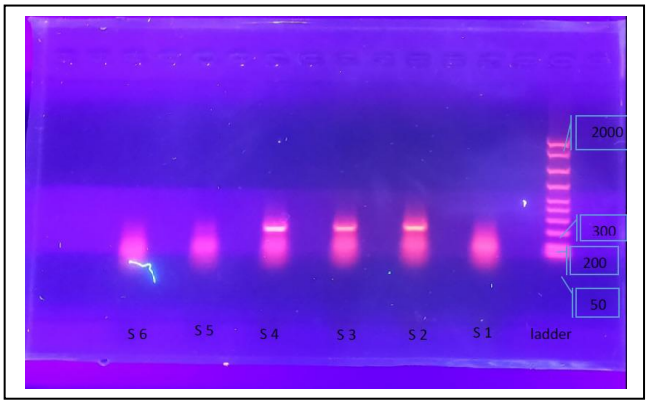

Figure 7. Gel electrophoresis showing a 223 bp band after purification and amplification of gene

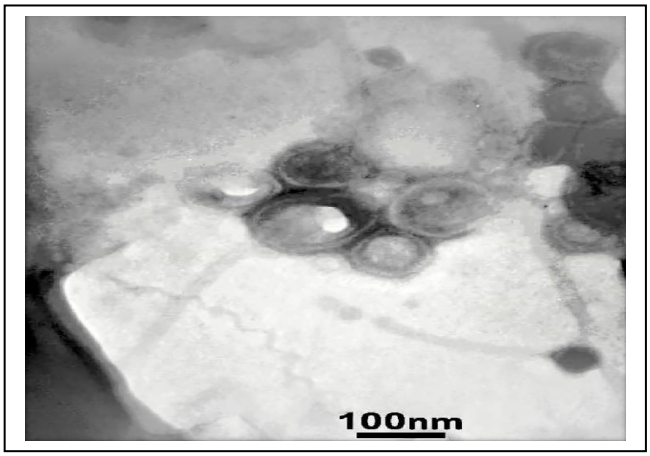

Figure 8. Electron microscope showed the morphology of feline infectious peritonitis virus crown-like appearance of isolated feline infectious peritonitis virus by using Electron Microscope

4. Discussion

The real-time RT-PCR assay which has been used in many studies for the amplification of S gene targeted to a highly conserved region of the viral genome (3' UTR), was used to detect the FIP virus (10-12) in the present research. To further elucidate the genetic characteristic of FCoV, both RNA samples from peritoneal fluid and fecal swabs were subjected to RT-PCR, and the spike genes were amplified (11). The result obtained from S-gene analyses revealed the presence of type-1 FCoV based on RT-PCR and virus isolation by cultivation.

The current study described the isolation and

detection of FCoV from cats presented with clinical evidence suggestive of FIP. The growth of local isolate of feline coronavirus in cell cultures fibroblast was determined by CPE detected in the first, second, and third viral passage. The infected cells were demonstrated to form a giant cell known as a syncytial cell. The cytopathic effects observed in cell culture fibroblast in this study are in agreement with previous reports (13-15). Since the CPE becomes more pronounced after 48 hours third passage post-inoculation, the infected fibroblast cells became rounded and detached from the surface of the tissue culture flask at 72 hours post-inoculation. Reports have suggested that FCoVs type I is difficult to grow in cell culture, and Fcwf-4 cell is more permissive (14, 16-18).

To further confirm the findings of the present study, an RT-PCR assay was used with primers targeted to a highly conserved region of the viral genome (3' UTR) (10, 12, 19). To further elucidate the genetic characteristic of FCoV, both RNA samples from ascetic fluid and infected cell cultures were subjected to RT-PCR, and the spike genes were amplified (11). The result obtained from S-gene analyses revealed the presence of type-1 FCoV based on RT-PCR and virus isolation (11, 20, 21).

In conclusion, this study for the first time in Iraq sought to isolate the Feline infectious peritonitis virus. The virus was found to be well adaptable in Fibroblast cell cultures and confirmed to belong to the type I serotype byS-gene amplification. The results of this study provide baseline information which could be used to further understand the pathogenesis and genetic diversity of FCoV in the domestic cat population.

Authors' Contribution

Study concept and design: O. M. I.
Acquisition of data: O. M. I.
Analysis and interpretation of data: H A. K.
Drafting of the manuscript: A. B. A.
Critical revision of the manuscript for important intellectual content: O. M. I., A. B. A. and H A. K.
Statistical analysis: H A. K.
Administrative, technical, and material support: O. M. I. and A. B. A.

Ethics

The study procole was approved by the ethics committee of University of Baghdad, Baghdad, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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