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Research Article

Feline herpes virus-1 (FeHV-1) in cats with ophthalmic problems: attempted propagation in CRFK cell lines

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ABSTRACT

Feline herpesvirus-1 (FeHV-1) is classified within the Varicellovirus genus and is frequently seen in cats. Ocular complications, such as conjunctivitis, keratitis, and corneal ulcers, are common and have the potential to result in latency and permanent visual loss if not appropriately diagnosed and monitored. This study aimed to isolate FeHV-1 from cats with ocular lesions using the CRFK cell line. This study included a total of ten cats that tested positive for FeHV-1 using PCR and showed symptoms, including ocular and nasal discharge (8/10), conjunctivitis (6/10), and keratitis (5/10). Conjunctival samples were collected and processed for nucleic acid extraction. The CRFK cell line was propagated, and all positive samples were inoculated in 6-well plates. Interestingly, no CPE was observed in the CRFK cell cultures during the observation period. Following post-inoculation in cell culture, the PCR analysis conducted on the supernatants obtained from the cultures found negative for FeHV-1. This study points out the challenges faced in isolating FeHV-1 in the CRFK from ocular samples of naturally infected cats. This highlights the requirement for future comprehensive in vitro studies to enhance the efficacy of FeHV-1 isolation techniques and explore potential approaches for FeHV-1 diagnosis.

INTRODUCTION

Feline viral rhinotracheitis (FVR) can manifest itself clinically in a variety of ways, including sneeze, nasal discharge, ocular discharge, conjunctivitis, coughing, oral ulcers, anorexia, fever, and lethargy (Gaskell et al. 2007; Magouz et al., 2022; Stiles 2014). *Feline herpesvirus type 1* (FeHV-1), *feline calicivirus* (FCV), *Chlamydia felis*, and more recently, *Bordetella bronchiseptica* (*B. bronchiseptica*) and *Mycoplasma felis* (*M. felis*) are the most common pathogens related with FVR (Lister et al., 2015; Sykes and Shelley, 2013; Walter et al., 2020).

FeHV-1 is a significant viral pathogen that affects domestic cats worldwide. FeHV-1 is a DNA virus, belongs to the genus *Varicellovirus*, subfamily *Alphaherpesvirinae*, and family *Herpesviridae* and is the primary causative agent of FVR, a respiratory disease characterized by sneezing, nasal discharge, and ocular manifestations (Gaskell et al., 2007). It has also been reported that clinical symptoms such as fever, oculo-nasal discharge, conjunctivitis, keratitis, pneumonia, abortion and fetal death can be seen in cats in the infection caused by FeHV-1 (Lee et al., 2019). Ocular complications associated with FeHV-1 infections, such as conjunctivitis, keratitis, and corneal ulcers, are common and can lead to severe visual impairment if not appropriately managed (Stiles, 2014).

Cats with acute FVR may shed the agent from and any age, vaccinated or unvaccinated, can be occasionally affected by this infection (Povey, 1979; Sykes et al., 1997). Most infected cats will later go on to become carriers because of the infection's tendency to remain latent in the neural ganglia (Haid et al., 2007). FeHV-1 can remain latent especially in the tissues of the head (trigeminal ganglia, optic nerves, olfactory bulbs, cornea, throat, salivary gland, lacrimal gland, cerebellum and conjunctiva) (Burgesser et al., 1999).

Virus isolation in cell culture, pathological findings, direct immunofluorescence test, PCR and serological methods are used in the diagnosis of FeHV-1 (Lister et al., 2015; Sykes and Shelley, 2013; Tan et al., 2020). One of the most effective ways among these methods is the isolation of the agent in a susceptible cell culture. The CRFK (Crandell-Rees feline kidney) cell line is widely used for propagating various feline viruses, including FeHV-1, due to its susceptibility to infection and ability to support viral replication (Haid et al., 2007; Henzel et al., 2011). CRFK cells are derived from feline kidney tissue and therefore provide an environment that is more physiologically relevant to FeHV-1 compared to non-feline cell lines. This natural host compatibility can enhance viral replication and allow for the study of specific virus-host interactions. The cells maintain their susceptibility to FeHV-1 over extended

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periods, allowing for long-term studies and the establishment of persistent infections. CRFK cells provide an excellent platform for studying FeHV-1-induced cytopathic effects (CPEs) and viral morphogenesis (Gaskell et al., 2007). The cells readily support viral replication, leading to characteristic changes in cell morphology that can be visualized and analyzed using various microscopy techniques. As a consequence of long-term cultivation in vitro, CRFK cells may undergo genetic changes and acquire phenotypic alterations. These changes can influence the susceptibility to FeHV-1 infection, alter the expression of host factors, and potentially impact the interpretation of experimental results (Maes, 2012).

In this regard, ocular samples taken from cats with ophthalmic lesions were inoculated into CRFK. It was aimed to produce FeHV-1 by inoculating the inoculum prepared from the samples taken for our research into sensitive cell culture. For effective treatment methods and preventative measures against ocular disorders in cats, it is crucial to understand the mechanism of action and replication of FeHV-1 in CRFK.

MATERIALS and METHODS

Animals

Ten FeHV-1-positive cats of varying ages, breeds, and genders with ocular lesions were used in this study between 2021 and 2022. They were brought to the Virology Laboratory of Veterinary Medicine, the Animal Hospital of Burdur Mehmet Akif Ersoy University, and private veterinary clinics. Veterinarians of the patients provided information on the cats to which each sample belonged. Animals had a variety of severe symptoms, including oculo-nasal discharge, conjunctivitis, and keratitis. The same veterinarian conducted an ophthalmological examination on each animal and recorded its symptoms.

Samples

The samples were collected with sterilized commercial swab sticks dipped in antibiotic PBS. All of the liquid in the swab sticks was transferred to sterile 2 ml microtubes after entirely mixing with a vortex. It was centrifuged for 20 minutes at +4 °C at 3000 rpm. After centrifugation, 500 µl of the supernatant was taken and kept at -80 °C until tests.

Nucleic acid extraction and PCR test

The resulting supernatants were extracted with a commercial viral nucleic acid isolation kit (Roch, Germany). Post-extraction PCR test was performed in accordance with the method of Henzel et al., (2012). Samples found to be positive were aliquoted and stored at -80 °C until tests. An extract from a commercial vaccine that contains FeHV-1 was used as the positive control in the PCR test. As negative control ultrapure water was used.

Cell culture

In this study, a CRFK cell line susceptible to feline viral infections was used. As a cell culture maintenance medium, sterile commercial Dulbecco's Modified Eagle Medium (DMEM) with high glucose (4.5 g/l) was used. Prophylaxis included antibiotics (10000 units/ml penicillin, 10.00 mg/ml streptomycin, 9.00 mg/ml sodium chloride) and antimycotics (250 g/ml amphotericin B, 205 g/ml sodium deoxycholate). Sterile fetal calf serum (FDS) was used at a rate of 10% for cell culture research. CRFK cell cultures were propagated in 25 cm^2 (50 ml) and 75 cm2 (250 ml) flasks (Corning, USA). Cell growth media was sterile commercial DMEM High Glucose (4.5 g/l) with L-Glutamine containing 10% FDS, 10% antibiotic, and 7% antimycotic. The cells cultures were incubated at 37°C in a humidified 5% CO^2 atmosphere for 3-4 days before being used for experiments. The incubated cells were inspected daily using an inverted tissue culture microscope (Olympus, Japan).

Inoculation of positive samples in CRFK

Cells that had been propagated were transferred into 6-well plates. Pre-conserved samples were thawed immediately in a 37 °C water bath. On two plates, ten samples were inoculated. After passing through 0.22 m sterile filters, all samples were inoculated into cell culture. Each plate had a cell control (HK) well. The same volume of PBS as the inoculum was given to the cell control wells. Every well was tested daily for any signs of cytopathologic effects (CPE) for 5 days after inoculation. All plates were freeze-thawed at -80 and 37 °C at the end of the fifth day. All well fluids were transported to 15 ml centrifuge tubes. All tubes were centrifuged for 20 minutes at +4 °C at 3000 rpm. 2 ml of supernatant was taken from each tube for PCR testing. All samples were blind passaged twice in CRFK.

RESULTS

Symptoms

Ten FeHV-1-positive cats showed signs of oculo-nasal discharge (8/10), conjunctivitis (6/10), and keratitis (5/10), either individually or in multiple combinations (Table 1) (Fig 1).

Cell culture

Table 1. Clinical signs of cats with FeHV-1 positive.

Symptoms	Numbers	Percentage	Total
Oculo-nasal discharge	8	%80	10
Conjunctivitis	6	%60	10
Keratitis	5	%50	10

Frozen samples were allowed to thaw in a 37 °C water bath for maximum progress. Both 6-well plates were seeded with the CRFK cell line. A single well on each plate was assigned as a cell control. However, there were no cases of CPE detected within the observed time period, although all samples were blind-passed twice in CRFK (Fig 2).

Post-inoculation (PI) PCR test

A total of ten positive swab samples were seeded onto a six-well plate that had been coated with CRFK. After five days PI, the supernatants from those cultures were collected and used for DNA extraction. We used the same PCR method described in the materials and methods section to check these samples for FeHV-1 nucleic acid. There were no positive results seen in any of the samples. (Fig 3).

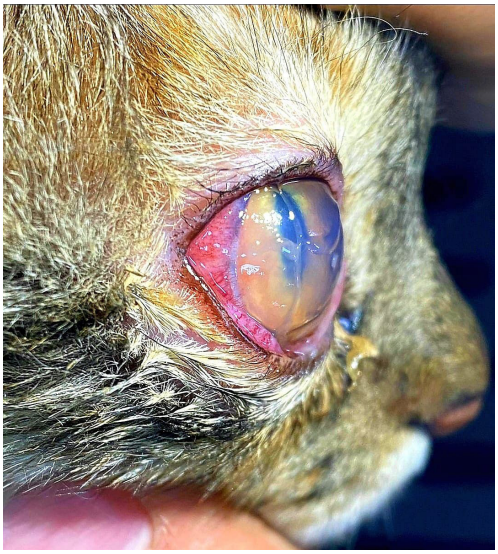

Figure 1. FeHV-1 positive cat (No. 2) with complex conjunctivitis and keratitis symptoms.

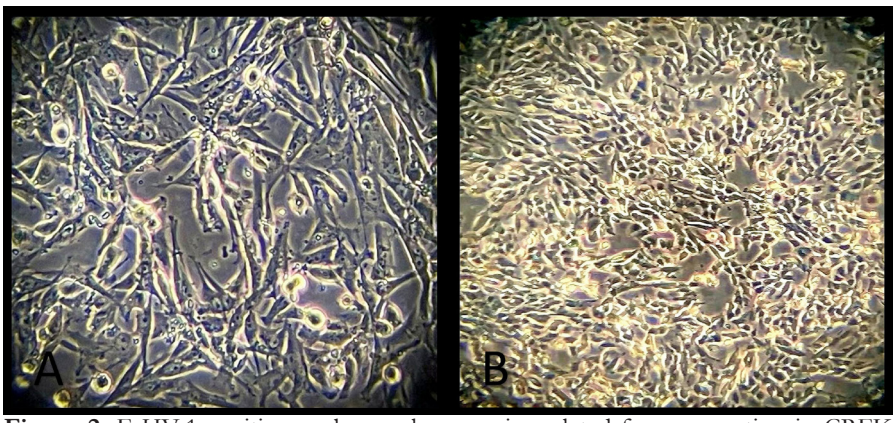

Figure 2. FeHV-1 positive swab samples were inoculated for propagation in CRFK cell lines. No CPE were detected. (A) Control, uninfected CRFK cells at 24 h. 10x magnification (B) CRFK cells inoculated with sample no: x at 120 h post-inoculation. 4x magnification. Olympus CKX41 inverted microscope (Olympus Corporation, Tokyo, Japan).

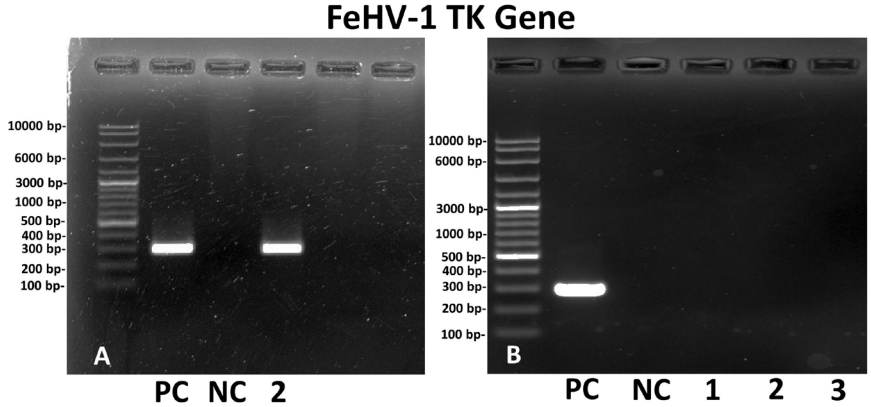

Figure 3. A conducted PCR test result before and after being inoculated, shown in the gel electrophoresis image. The swab sample collected from cat No. 2, which exhibited clinical symptoms including noticeable discharge from the eyes and nose as well as conjunctivitis, yielded a positive result prior to inoculation (A. before to inoculation; B. after inoculation). PC: FeHV-1 contained vaccine extract. NC: Ultrapure water.

DISCUSSION

FeHV-1 infects cats leading to in a wide range of clinical signs, including but not limited to sneeze, nasal discharge, ocular discharge, conjunctivitis, coughing, oral ulcers, anorexia, fever, and lethargy (Stiles, 2014). PCR detection of FeHV-1 can be routinely performed in oculo-nasal secretions from conjunctival or nasopharyngeal swab, and in uncoagulated blood (Walter et al., 2020). In most cases, these samples should be tested at the same time since their integrated results can be useful in the diagnosis of FeHV-1 infection (Sykes and Shelley, 2013). For pharynx, tongue, nose and conjunctiva samples, a recent study has shown that FeHV-1 tended to be most frequently detected in the nose, but this difference was not significant compared with other locations (Schulz et al., 2015). FeHV-1 infection causes acute upper respiratory and ocular disease, which is particularly severe in kittens. The tonsils, conjunctiva, and nasal mucosa are the locations where FeHV-1 predominantly infects muco-epithelial cells (Gould, 2011). So, oronasal and conjunctival swab extracts are often used as samples for virus isolation or PCR to diagnose acute FeHV-1 in the lab. It is thought that corneal dendritic ulcers are the pathognomonic sign of FeHV ocular infection (Maes, 2012). Almost all cats experience a latent infection following the acute phase of the disease, which can occasionally reactivate and result in recurring clinical symptoms, mostly ocular infections like keratitis and conjunctivitis. Hence, the use of conjunctival swabs has significant value in the detection and isolation of FeHV-1 (Henzel et al., 2011; Litster et al., 2015). This study included cats with a high prevalence of oculo-nasal discharge (80%), however because of their anatomy, we were only able to collect the conjunctival swab samples. This has also been highlighted as a concern by previous research (Veir et al., 2008).

Traditional methods involve the inoculation of clinical samples onto susceptible cell cultures, followed by observation for CPE. Low viral loads can present challenges for virus isolation as they may not contain sufficient infectious viral particles to establish productive infection in cell cultures. Despite the detection of viral nucleic acids through PCR-based assays, the presence of low viral load can lead to the failure of virus isolation. Studies have shown that samples with low viral loads often exhibit weak or no CPE in cell cultures, limiting the ability to isolate infectious virus (Leland and Ginocchio, 2007). Accurate quantification of viral load in clinical samples could help determine the likelihood of successful virus isolation. Cats with eye problems were included in our study, however it is possible that the swab samples we collected did not contain enough infectious viral particles to be propagated in CRFK cell culture. The likelihood of successfully isolating the virus might be improved by using a procedure involving two or more blind passages (Haid et al., 2007). There are a few reasons why PCR is a more sensitive method for identifying FeHV-1 in oculo-nasal secretions than traditional tissue culture isolation methods (Burgesser et al., 1999; Sandmeyer et al., 2010). This could also be an explanation of why we were unable to grow virus in CRFK in our study, although all samples detected positive by PCR. Transport, freezing, thawing, and the enzymes in saliva and tears might have all been involved in the decomposition of the herpesvirus envelope. It's possible that the antibodies complexed with the virus in the tears and saliva, reducing the virus's infectivity. It's also probable that many of the infectious virions released in the saliva and tears are defective in maturation (Reubel et al 1993).

The cell culture method used is crucial for effective virus isolation, for FeHV-1 isolation depends on the specific strain or isolate being studied. Different cell lines have been used for successful FeHV-1 isolation, and their suitability may vary (Leeming et al., 2006; Sun et al., 2014; Yang et al., 2020). CRFK cells, derived from feline kidney tissue, are one of the most widely used cell lines for FeHV isolation (Walter et al., 2020). In our study, the failure to isolate FeHV-1 was unexpected; there is a well-described carrier state in FeHV-1 infections, and they appear to be widespread. Permanent cell lines used for viral propagation may not always support the replication of a given virus strain. Some strains have strict tropism and may require specific cell types expressing appropriate receptors for productive infection (Yang et al., 2020). If the selected cell line lacks the necessary receptors or host factors, the virus may fail to establish infection, despite the detection of viral nucleic acids in the clinical sample. FeHV-1 exhibits a natural tropism for epithelial cells, particularly those of the respiratory tract (Leeming et al., 2006). However, in some cases, FeHV-1 may establish a non-productive or latent infection in cell culture systems, leading to the absence of observable CPE (Cannon et al., 2010). This non-productive infection can hinder successful virus isolation, as it becomes difficult to distinguish between true virus isolation failure and latent infection. On the other hand, while other pathogens such as *B. bronchiseptica* have the potential to be a primary agent, they are more likely to contribute to FVR as a secondary or opportunistic infection in the clinical setting (Sykes and Shelley, 2013; Walter et al., 2020). We had to abandon comprehensive pathogen identification for this study due to a lack of resources. The results could indicate an uncommon FeHV-1 infection in cats, but further work is necessary to confirm these preliminary findings.

Cellular factors present in the clinical sample may inhibit viral replication, thereby preventing the successful isolation of infectious virus. Various components such as antiviral proteins, low pH, or lack of some essential amino acids can negatively impact FeHV-1 replication in CRFK (Maggs et al., 2000; Storey et al., 2002). These factors may not be adequately accounted for during PCR-based detection, leading to the false-positive result without successful virus isolation. In our research, we used commercial DMEM supplemented with arginine and lysine at 84 and 146 mg/L, respectively. By optimizing these amino acid concentrations in this medium, positive results may be obtained for isolating FeHV-1 in CRFK (Bol and Bunnik, 2015).

Virus isolation is a crucial step in understanding the pathogenesis, epidemiology, and potential therapeutic interventions for viral infections. Feline viruses have long been studied and isolated using cell culture techniques (Sykes and Shelley, 2013). Although cell culture techniques are widely used, viral adaptation needs virus isolation from persistent cell cultures, which has shown to be challenging or unsuccessful (de Parseval et al., 2004). Virus culture, isolation, and identification of FeHV-

1 from pharynx, tongue, nose and conjunctiva or buffy coat samples is strongly supportive of a diagnosis of FVR in cats with compatible clinical signs. The duration for virus culture and identification in a laboratory can be as minimal as 4-5 days when the sample has an adequate viral load. However, due to the lengthy procedure of tests completion, these tests are of limited practical use in rapid diagnosis of disease.

CONCLUSION

The objective of the study was to successfully and rapidly isolate FeHV-1 from cats that were naturally infected and showed eye problems. In light of unexpected results, our investigation focused at exploring the factors associated with struggling to isolate FeHV-1 in the CRFK cell line. Finally, we tried to clarify the potential underlying causes behind the limitations. In conclusion, further studies are necessary in in vitro systems to effectively isolate FeHV-1 and exploring potential control and treatment strategies.

DECLARATIONS

Ethics Approval

All procedures were approved by the Animal Ethics Committee (AEC) Burdur Mehmet Akif University, Türkiye (No:102/914).

Conflict of Interest

Authors do not have any conflict of interests

Consent for Publication

Consent on publication was comfirmed with approval from the Republic of Türkiye Ministry of Agriculture and Forestry, Directorate of Burdur Provincial (No: E-69877819-325.04.02-6073614).

Competing Interest

The authors declare that they have no competing interests

Author contribution

Idea, concept and design: HSS, YF

Data collection and analysis: HSS, YF

Drafting of the manuscript: HSS

Critical review: HSS

Data Availability

Not applicable.

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