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Basic biological characterization of feline morbillivirus

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ABSTRACT. Feline morbillivirus (FmoPV) is an emerging virus that was recently discovered in domestic cats with chronic nephritis. Despite the potential role of FmoPV in chronic nephritis, little is known about its biological characteristics. In this study, we established a quantitative assay of FmoPV by using an indirect immunofluorescence technique. Viral titers of FmoPV were determined in one week. Treatment with polybrene® or trypsin which was previously used in virus isolation did not augment the virus titers. FmoPV was notably stable at 4°C, retaining high titers for at least 12 days. Heat-treatment at 60°C and 70°C effectively inactivated FmoPV in 10 and 2 min, respectively. The biological characteristics of FmoPV reported here will be beneficial for establishing an efficient virus isolation method and will provide important information to take a measure to reduce the risk of FmoPV infection.

KEY WORDS: feline, feline morbillivirus, quantification, replication kinetics, stability

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Feline morbillivirus (FmoPV) is a new morbillivirus in domestic cats that was first isolated in Hong Kong in 2012. FmoPV is a pleomorphic, enveloped, negative-sense single-stranded RNA virus belonging to the family *Paramyxoviridae* [17]. Other morbilliviruses, including measles virus (MV), canine distemper virus (CDV) and rinderpest virus, cause a number of diseases with a broad host range [6].

Approximately 30% of old cats aged over 15 years suffer chronic renal failure [7], and chronic renal failure is one of the major clinical concerns for aged cats. Tubulointerstitial nephritis is the most common morphologic diagnosis for chronic renal failure in cats [3]. Importantly, FmoPV is considered to be associated with tubulointerstitial nephritis [5, 17]. In Japan, FmoPV RNA was detected in kidney tissues of cats with nephritis, with a positive rate of 40% [5]. However, it is still obscure whether the FmoPV infection causes tubulointerstitial nephritis in cats.

FmoPV infection is prevalent at least in Hong Kong and Japan. A previous report by Woo *et al*. showed that FmoPV RNA was detected in 56 (12.3%) of 457 stray cats by reverse transcription (RT)-PCR test [17]. In Japan, when 82 urine and 10 random blood samples of cats were tested by RT-PCR, 5 urine samples and one blood sample (6.1 and 10%) were positive for FmoPV RNA, respectively [5].

In spite of the potential importance of the virus in veterinary field, the virus isolation has been succeeded only in 2 research groups [12, 17]. Woo *et al*. isolated FmoPV after very long incubation period (presumably 16 to 24 weeks according to the literature) [17], whereas we succeeded in isolating the virus from fresh urine samples of 3 FmoPV-positive cats only in 2 to 3 weeks using Crandell-Rees feline kidney (CRFK) cells. *L*-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin and polybrene® (hexadimethrine bromide) were used in virus isolation in both groups; however, the effects of the treatments with these reagents on efficacy of virus isolation were unknown.

There have been no reports on the biological characteristics of FmoPV in the previous studies. Biological characterization of the virus may be useful for developing more efficient virus isolation methods and will give insights for reducing the chances of FmoPV infection. In the present study, we established a quantitative assay for infectious FmoPV by an indirect immunofluorescence (IF) technique. After establishing the titration method, we examined the effects of trypsin and polybrene® on virus titers. Then, the replication kinetics of FmoPV was examined in CRFK cells. We further determined the stability of FmoPV at various environmental temperatures and evaluated the efficacy of heat inactivation at 60°C and 70°C.

MATERIALS AND METHODS

Cells: CRFK cells (domestic cat kidney) (ATCC, CCL-94) were cultured in a growth medium of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 IU/m*l*) and streptomycin (100 μg/m*l*) (Invitrogen, Carlsbad, CA, U.S.A.) at 37°C in a humidified atmosphere with 5% CO_2 in air.

Virus: Stock viruses of FmoPV strain SS1 [12] were prepared from the culture supernatant of CRFK cells persistently infected with the strain (termed CRFK/FmoPV cells). All stock viruses were sonicated and kept at −80°C until used, except for titration without freeze and thaw.

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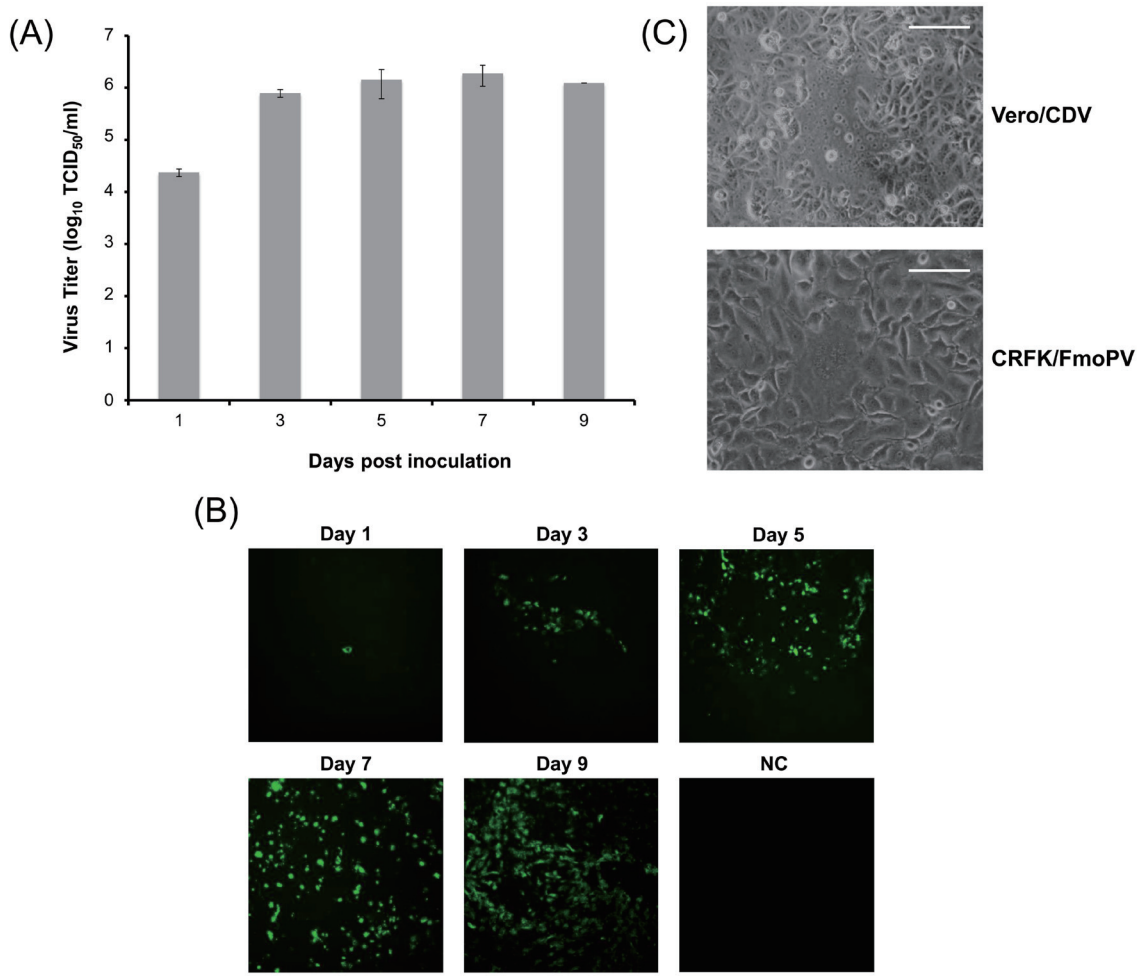

Fig. 1. Determination of incubation period for obtaining the maximum titer of the stock virus. (A) The titers of stock virus of FmoPV strain SS1 at different incubation periods. The data are the means ± standard deviations of three experiments. (B) Green cytoplasmic fluorescence in FmoPV-inoculated CRFK cells. At 9 d.p.i., partial detachment of cells was also observed. (C) Comparison of CPEs of FmoPV-infected CRFK cells and CDV-infected Vero cells. White bar: 200 μm.

Virus titration: Virus titration was carried out in a 96 multi-well plate. Three-fold serially diluted FmoPV strain SS1 was distributed at 50 μl per well, and 4 replicates per dilution were tested on a 96 multi-well plate. Then, 50 μl of CRFK cells (5×10^3 cells) were added to each well. After incubation at 37°C in a CO_2 incubator, virus-inoculated CRFK cells were fixed with 4% paraformaldehyde at room temperature for 10 min and quenched with phosphate-buffered saline (PBS) containing 100 mM glycine for 5 min. To detect viral antigens, indirect IF assay was conducted using the rabbit anti-FmoPV N protein antibody prepared as described previously [12]. The fixed cells were incubated with the diluted (1:2,000) anti-FmoPV N antibody, followed by a diluted (1:2,000) anti-rabbit IgG conjugated with Alexa Fluor 488 (Life Technologies, Carlsbad, CA, U.S.A.). Green fluorescence in the cytoplasm of FmoPV-infected CRFK cells was observed with a fluorescence microscope. The 50% tissue culture infective dose ($TCID_{50}$) was calculated by the method of Behrens-Kärber.

Replication kinetics: To measure the virus growth, 6×10^4 CRFK cells grown in a 6 multi-well-plate were inoculated with FmoPV at a multiplicity of infection (MOI) of 1 $TCID_{50}$ and incubated at 37°C for 3 hr for virus adsorption. The inoculated-CRFK cells were washed three times with PBS and then cultured in 2 m*l* of fresh growth medium. The culture supernatants were collected for virus titration at the times indicated in Fig. 3A, and the serially diluted supernatants were distributed at 50 μl per well, and 12 replicates per dilution were tested on a 96 multi-well plate. The infectious titer was measured by the indirect IF assay as described above.

RESULTS

Establishment of a titration method using CRFK cells: Firstly, we determined the optimum incubation period required for virus titration. We inoculated the serially diluted

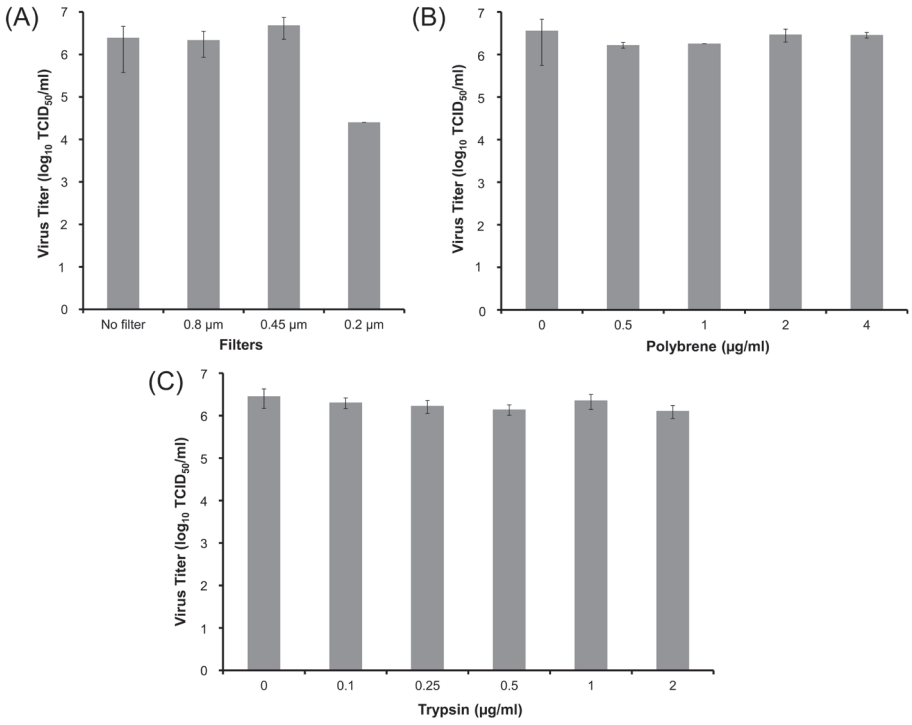

Fig. 2. Effects of filtration and treatments of polybrene® and TPCK trypsin. (A) Effect of filtration of FmoPV. Stock viruses were filtrated through 0.8, 0.45 or 0.2 μm membrane disc filters. (B) Effect of polybrene® treatment in FmoPV infection. Polybrene® was added to stock viruses at the concentration of 0.5, 1, 2 and 4μg/m*l*. (C) Effect of TPCK trypsin treatment in FmoPV infection. Stock viruses were treated with TPCK trypsin of different concentrations (0.1, 0.25, 0.5, 1 and 2 μg/m*l*) and incubated at 37°C for 1 hr. The data are the means ± standard deviations of three experiments.

stock virus into CRFK cells, and the virus-inoculated CRFK cells were incubated for 1, 3, 5, 7, 9 and 11 days at 37°C in a CO_2 incubator. Then, we determined the virus titer by indirect IF assay at each incubation period. As shown in Fig. 1A, the titer of FmoPV was elevated with incubation time and reached a plateau at 5 days post-inoculation (d.p.i.). Intensity of green cytoplasmic fluorescence increased up to 7 days post-inoculation (Fig. 1B). Scoring each well either IF positive or negative was relatively difficult at 5 d.p.i., and the end points became more apparent at 7 d.p.i. At 9 d.p.i., cells were detached partially (Fig. 1B). Therefore, we set the incubation period for 7 days in virus titration.

We previously reported that the infectivity of porcine endogenous retrovirus subgroup A (PERV-A) was significantly decreased by filtration through 0.2 μm membrane disc filters, although the diameter of PERV-A is considered to be approximately 0.1 μm [9]. Thus, we examined the effects of filtration against FmoPV. Reportedly, virions of FmoPV are variable in size, ranging from 0.13 to 0.38 μm in diameter [17]. To study the size of infectious FmoPV, stock viruses were filtrated through 0.8, 0.45 or 0.2 μm membrane disc filters (Acrodisc; PALL, Ann Arbor, MI, U.S.A.). Although virus titer after 0.2 μm filtration reduced by approximately 100-fold, filtration through 0.8 and 0.45 μm filter did not affect virus titers (Fig. 2A). The result indicated that the pleomorphic virions with a diameter of over 0.2 μm were infectious or many virions aggregated.

In some viruses, infectious titers are augmented by addition of polybrene® which assists viral adsorption in virus inoculation [11]. Stock viruses were inoculated into CRFK cells under the presence of various concentrations of polybrene® (0.5, 1, 2 and 4 μg/m*l*). Four hr after inoculation, growth medium containing polybrene® was replaced with fresh growth medium. The results showed that polybrene® did not augment titers of FmoPV (Fig. 2B).

Infections of most morbilliviruses including CDV are enhanced by treatment with trypsin. The inactive precursor F_0 protein of morbilliviruses is cleaved into the biologically active F protein consisting of F_1 and F_2 subunits required for membrane fusion in virus infection [15, 16]. To know the effect of trypsin treatment on FmoPV infection, TPCK trypsin (Sigma–Aldrich, Zwijndrecht, The Netherlands) (0.1, 0.25, 0.5, 1 and 2 μg/m*l*) was added to stock viruses. After incubation at 37°C for 1 hr, we added 100 μl of growth medium containing 20% FCS and antibiotics to each well. As a result, no significant effect on virus titers was observed after treatment with TPCK trypsin (Fig. 2C).

Replication kinetics of FmoPV: We then examined the replication kinetics of FmoPV. We inoculated FmoPV into CRFK cells at a MOI of 1. Although we washed virus in-

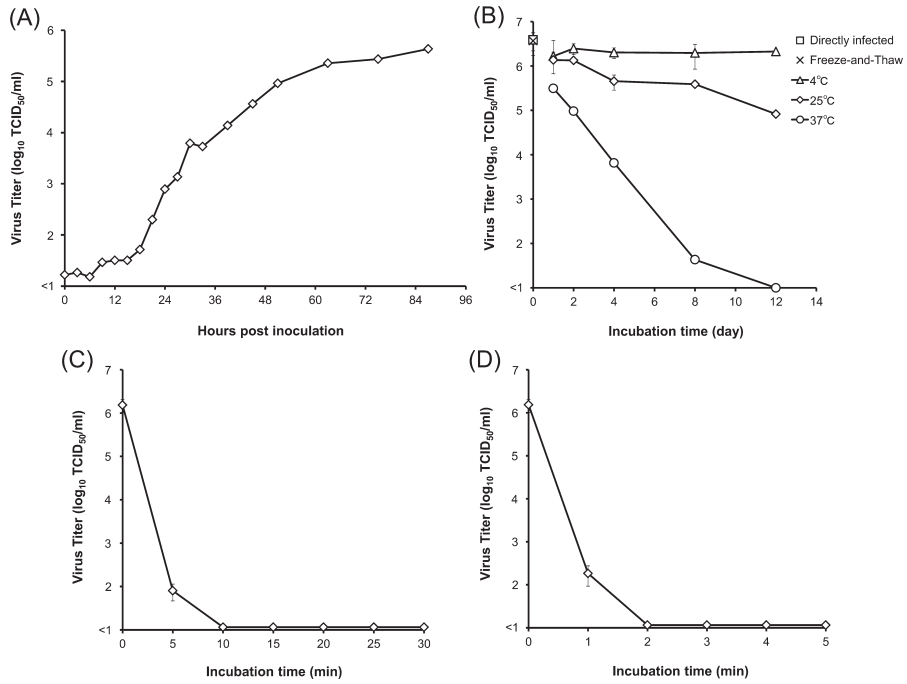

Fig. 3. Replication kinetics and thermal stability of FmoPV. (A) Replication kinetics of FmoPV in CRFK cells. CRFK cells were inoculated with FmoPV at a MOI of 1 $TCID_{50}$. After incubation at 37°C for 3 hr, the infected CRFK cells were washed three times and cultured in growth medium. The culture supernatants were collected at the indicated times and then titrated. (B) Thermal stability of FmoPV at 4°C, 25°C or 37°C. The data also show the comparison of virus titers with directly infected and freeze-and-thaw infected samples. The data are the means ± standard deviations of three experiments. Inactivation of FmoPV by heat treatment at 60°C (C) and 70°C (D). Stock viruses were incubated for indicated time up to 30 min in water baths with temperatures of 60°C and 70°C, respectively. The data are the means ± standard deviations of three experiments.

ocula three times after virus adsorption, residual viruses were detected at 0 hr post-infection (h.p.i.). The increase of virus titer was firstly observed at 9 h.p.i. From 18 to 30 h.p.i., the virus titer increased exponentially. Then, the increase of virus titer decelerated from 33 h.p.i., and almost no increase of virus titer was observed from 63 h.p.i. (Fig. 3A).

Stability of FmoPV at different temperatures: Next, we examined the ability of FmoPV to survive at different environmental temperatures. Stock viruses were incubated at 4°C, 25°C or 37°C for 1, 2, 4, 8 and 12 days. The virus titers after incubation were compared with titers of directly infected and freeze-and-thaw infected samples. As shown in Fig. 3B, freeze-and-thaw did not affect virus titers. The virus titers of FmoPV incubated at 37°C declined gradually and reached undetectable level at 12 d.p.i. The virus titers of FmoPV incubated at 25°C also decreased, but more mildly than at 37°C. The virus titers of FmoPV incubated at 4°C were maintained for at least 12 days.

Heat-inactivation of FmoPV: Finally, we evaluated the effect of heat treatment on FmoPV in eliminating the viral infectivity. Stock viruses were incubated for indicated time up to 30 min and 5 min in water baths with temperatures at 60°C and 70°C, respectively. The viruses were immediately chilled in an ice-water bath to stop the effect of heating and then inoculated into CRFK cells. As shown in Fig. 3C, FmoPV incubated at 60°C decreased its infectivity in 10 min. Increasing the temperature to 70°C greatly increased the rate of inactivation, and FmoPV was dramatically inactivated in 2 min (Fig. 3D).

DISCUSSION

In this study, we established the quantitative assay using an indirect IF technique for biological characterization of FmoPV. Like other paramyxoviruses, FmoPV causes characteristic cytopathic effects (CPE) with syncytia formation in cultured cells upon infection [8, 10, 12]. CPE can be observed under a light microscope at 3–4 d.p.i.; however, CPE induced by FmoPV is relatively small and unclear. In Fig. 1C, we show the CPE of CRFK cells induced by FmoPV strain SS1 and that of Vero cells (ATCC: CCL-81) induced by CDV strain Snyder Hill [14]. By indirect IF assay, we clearly detected green cytoplasmic fluorescence in FmoPV-infected CRFK cells using a rabbit anti-FmoPV N antibody [12]. Thus, the virus titration using the indirect IF technique is useful to determine the infectious titer of the FmoPV.

Treatments with polybrene® and trypsin seemed not to be necessary for virus titration and isolation. Other morbil-

liviruses use trypsin for cleavage of F protein [15]. Although immunoblot analysis showed that FmoPV F protein may be cleaved into F_1 and F_2 proteins [12], FmoPV F protein has only one basic proteolytic cleavage site, while the cleavage sites in other morbilliviruses are multibasic [17]. Based on the amino acid difference from other morbilliviruses, the F protein of FmoPV is likely to be cleaved by using a different peptidase.

FmoPV was found to be relatively stable at environmental temperatures. As shown in Fig. 3B, incubation at 37°C resulted in a gradual decrease in FmoPV infectivity, but FmoPV retained its infectivity for 12 days. The virus titer of FmoPV incubated at 25°C declined more mildly than at 37°C. The stability of FmoPV to survive environmental temperatures might facilitate virus spread in the field. FmoPV was remarkably stable at 4°C, without any significant loss of virus titers for at least 12 days. Thus, its high stability at 4°C may favor long-term storage of the specimens for virus detection and isolation.

Heat-treatment is one of the most common viral inactivation methods that is effective for both enveloped and non-enveloped viruses [2]. The mechanism of viral heat-inactivation is disassembly of virus particles into single proteins or noninfectious viral subunits [13]. All the members of family *Paramyxoviridae* are known to be relatively sensitive to heating [4]. Previous studies have shown that MV and Newcastle disease virus are effectively inactivated at 56°C in 30 and 10 min, respectively [1, 18]. In the current study, incubation at 60°C for 10 min reduced the viral titers to undetectable levels. (Fig. 3C). Heat treatments at higher temperatures led to a more rapid decline in viral titers. At 70°C, infectivity dropped dramatically and inactivated to undetectable levels in 2 min (Fig. 3D). From these data, we conclude that heat-treatment at 70°C is a reliable measure for inactivating FmoPV to minimize the risk of infection and contamination of FmoPV.

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