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Equine immunoglobulin F(ab’)2 fragments protect cats against feline calicivirus infection

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ABSTRACT

Feline calicivirus (FCV) causes upper respiratory tract infections in felines and threatens the health of wild and domestic felines. Clinically, specific drugs to treat FCV have not yet been developed. Here, IgG was extracted from inactivated FCV-immunized horse sera. Equine F(ab’)2 fragments were obtained from pepsin-digested IgG and then purified by protein-G column chromatography. In our study, equine immunoglobulin F(ab’)2 fragments showed efficient neutralizing activity in vitro against FCV and had therapeutic and prophylactic effects in FCV-infected cats. The anti-FCV-specific F(ab’)2 fragment can significantly alleviate the clinical symptoms of FCV-infected cats and reduce the viral loads of the trachea, lung and spleen. These results indicate that the F(ab’)2 fragment prepared from inactivated FCV-immunized horses may be used as a prophylactic and therapeutic agent for diseases caused by FCV.

1. Introduction

The feline calicivirus (FCV) belongs to the family Caliciviridae and is a respiratory pathogen widely prevalent in felines. Cats, tigers, cheetahs and other wild felines are susceptible, kittens aged 6 to 84 days are mainly infected, with a mortality rate of 67% [1,2]. After FCV infection, the animals have oral ulcers, chronic stomatitis, rhinitis, conjunctivitis and pneumonia. FCV is distributed worldwide and is highly contagious in felines. Once the kittens are infected, FCV proliferates in the oral cavity and upper respiratory tract, causing feline upper respiratory tract disease (FURTDD) [3]. The clinical symptoms are relieved 14 to 18 days after the infection, but the FCV is still excreted through saliva and nasal secretions, which is the main reason for the substantial spread of FCV [3].

There have been many reports on anti-FCV drugs in recent years. Hongxia Wu et al. discovered the inhibitory effects of germacrone and LiCl on FCV [4,5]. In addition to synthetic compounds, Korea red ginseng extracts (KRGE) and ginsenosides, grape seed extracts (GSE), ginger extracts, clove extracts and phaeophyta extracts can also resist FCV [6–9]. In past studies, equine IgG was gradually favored by researchers as a blood-derived biological product that is easy to obtain in large quantities and has a low cost. Antibodies, particularly F(ab’)2 fragments, are easy to purify in large quantities and are inexpensive and have been reported to prevent Rabies Virus (RV) and West Nile Virus (WNV), Middle East respiratory syndrome coronavirus (MERS-CoV), Ebola Virus (EBOV) [10–13].

Here, we used inactivated-FCV-immune horses to prepare hyper-immune serum and extract IgG. Thereafter, the F(ab’)2 fragments were produced from the IgG digested by pepsin to avoid potential allergic reactions between different species of animals. We evaluated the protective effects of equine IgG (in vitro) and F(ab’)2 fragments (in vitro and in vivo) on FCV. First, we determined the neutralization titer of the IgG. The F(ab’)2 fragments were produced from inactivated-FCV-immunized horse sera. Equine F(ab’)2 fragments were obtained from pepsin-digested IgG and then purified by protein-G column chromatography. In our study, equine immunoglobulin F(ab’)2 fragments showed efficient neutralizing activity in vitro against FCV and had therapeutic and prophylactic effects in FCV-infected cats. The anti-FCV-specific F(ab’)2 fragment can significantly alleviate the clinical symptoms of FCV-infected cats and reduce the viral loads of the trachea, lung and spleen. These results indicate that the F(ab’)2 fragment prepared from inactivated FCV-immunized horses may be used as a prophylactic and therapeutic agent for diseases caused by FCV.

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that F(ab')2 reduced FCV in tissues. These results suggest that equine neutralizing F(ab')2 fragments may protect cats against FCV pathogenic infection.

### Table 1

| Clinical symptoms | Description | Score |
|-------------------|-------------|-------|
| Rectal temperature (°C) | < 37.0 | 0 |
| | 37.1–39.4 | 1 |
| | ≥ 39.5 | 2 |
| Body weight loss (%) | Gain or loss of < 3% | 0 |
| | Loss of ≥ 3% | 2 |
| Ulcers (oral and/or nasal) | Absence | 0 |
| | Small and few | 1 |
| | Large or numerous | 3 |
| Nasal discharge | Absence | 0 |
| | Slight | 1 |
| | Copious | 2 |
| Ocular discharge | Absence | 0 |
| | Presence | 1 |

2. Materials and methods

2.1. Antigen preparation

F81 cells were infected with FCV (CH-JL 2 strain isolated by Yanli Zhao) at a multiplicity of infection (MOI) of 0.5 \[ 14 \]. Culture supernatants were harvested at 24 h post infection, then frozen and thawed three times. After inactivation with 4% formaldehyde, the culture supernatants were centrifuged at 3000 × \( g \) for 45 min to remove cell debris. The clarified supernatants were ultracentrifuged at 150,000 × \( g \) for 1.5 h at 4 °C. The precipitate was dissolved in phosphate-buffered saline (PBS) and added to a 10-30-50% sucrose gradient of varying concentrations.

2.2. Horse immunization

Three 3- to 4-year-old healthy black horses received multipoint intramuscular injections in the backside with 1.5, 2.5, 3.0, 5.0, 5.0 and 5.0 mg of FCV emulsified in Freund’s complete adjuvant (the first two immunizations) or Freund’s incomplete adjuvant (the last four immunizations) at weeks 0, 1, 2, 3, 5 and 7. These horses were provided by the Military Red Mountain Stud Farm (Changchun, China). The sera were collected from the jugular veins 2 weeks after each injection and stored at −20 °C.

![Fig. 1. Horse serum-specific IgG neutralization titers and evaluation of specific IgG and F(ab')2.](image-url)

(A) Horse sera were collected two weeks after each immunization, and the neutralizing titer of each collected serum sample was determined. IgG in horse serum was precipitated by ammonium sulfate solution, digested with pepsin to produce F(ab')2, and further purified by a protein-G column. (B) SDS-PAGE with pF (ab')2 and IgG were stained with Coomassie blue. (C) The purity of the pF (ab')2 fragment was 95.236% according to thin-section scanning.
2.3. Immunoglobulin purification

The horse sera were diluted to a concentration of 50% with PBS, and then a saturated ammonium sulfate solution was added until the ammonium sulfate concentration was 50%. The solution was allowed to stand at 4 °C for 3 h and then centrifuged at 5000 × g for 20 min. After removing the supernatant, the precipitate was dissolved in PBS, and saturated ammonium sulfate was added until the concentration of ammonium sulfate was 33%. The solution was allowed to stand at 4 °C for 3 h and then centrifuged at 5000 × g for 20 min. After repeating the 33% ammonium sulfate step once, the precipitate was dissolved in PBS and dialyzed against PBS at 4 °C for 18 h to remove the ammonium salt.

2.4. F(ab’)2 purification

After pepsin (Promega, USA) was activated with a NaAc solution, double-diluted IgG was adjusted to PH 3.4 with 1 mol/L of HCl. A final concentration of 10,000 IU/mL activated pepsin was added to the diluted IgG solution. After reacting at 37 °C for 2 h, 1 mol/L NaOH was added to adjust the pH to 7.2. This solution was further purified by a protein-G column (GE Healthcare, USA). The purified protein was then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining. The purity of F(ab’)2 fragments was analyzed with a CS-9301 thin-layer chromatography scanner (Shimadzu, Japan), and the protein was stored at 4 °C for further use.

2.5. FCV neutralization assay

The F(ab’)2 fragments and equine serum neutralization titers were detected in the FCV-infected F81 cells. Heat-inactivated equine serum samples, purified IgG or F(ab’)2 were diluted initially to 1 mg/mL as the highest dose and then by two-fold serial dilutions in DMEM (1 ×, Sigma, USA) with 5% FBS (GIBCO, USA). To each well (from 1st to 9th column) containing 50 μL of serially diluted sample, 50 μL of FCV containing 200 times the half-tissue culture infectious dose (TCID₅₀) was added. The 10th column well of each plate was kept as a virus control containing 50 μL of DMEM (1×) with 5% FBS and 50 μL of FCV (200 TCID₅₀) suspension. The 11th and 12th column wells were kept as normal cell controls. After 1 h, 100 μL of F81 cell suspension containing approximately 1.5 × 10⁵ cells was added. The plate was incubated at 37 °C in a 5% CO₂ atmosphere, and the final results were recorded. The 50% neutralization end point of the samples was calculated by using the Reed and Muench formula.

2.6. Indirect immunofluorescence assay (IFA)

Monolayer F81 cells were inoculated with FCV (MOI = 0.1). After
24 h, DMEM was removed for 30 min with 80% cold acetone (−20 °C). Then, the cells were washed 5 times with PBS containing 0.05% Tween-20 (PBST), and the F(ab')2 fragments diluted with 10% BSA were added and incubated for 1 h in a 37 °C incubator. After washing 5 times with PBST, 200-fold diluted FITC-labeled rabbit anti-horse IgG secondary antibody was added to the cells (Bioss Antibodies, China). Light was avoided, and the solution was incubated in a 37 °C incubator for 1 h. Fluorescence was observed under a Leica DMi8 inverted fluorescence microscope after a PBST wash (Leica, Germany).

2.7. Prophylactic and therapeutic efficacy of F(ab')2 fragments against FCV in cats (in vivo)

Healthy female cats, 6–8 weeks old and weighing between 0.5 and 0.6 kg, were randomly divided into groups of 4 cats. Cats were purchased from the pet market (Changchun, China). To investigate the preventive role of F(ab')2 against FCV infection, the animals (4 cats per group) were injected on day −1 (the day before viral infection) subcutaneously with 1, 2 or 5 mg/kg of purified horse immune F(ab')2 or an equal volumes of normal horse F(ab')2 as a negative control. Twenty-four hours later (day 0), the cats were infected intranasally with FCV (containing 10^7.67 TCID50) in a total volume of 500 μL DMEM. To evaluate the therapeutic role of F(ab')2 against FCV infection, the treatment was given subcutaneously at 1–3 days post infection (dpi) with 2, 5 or 10 mg/kg purified horse immune F(ab')2 per cat. The control group was given equal volumes of normal horse F(ab')2 as the mock treatment. Clinical signs and survival rates were monitored in all animals, and body weights and rectal temperatures were measured every day until 14 dpi. The cats’ health were rated on days 0 and 7 (Table 1 for details) [15]. Virus titers of oral swabs were detected by quantitative real-time reverse transcription-PCR (qRT-PCR).

2.8. Viral load and virus shedding assay

A cat in each group was euthanized at 14 dpi. Lungs, tracheas, kidneys, livers and spleens were harvested from cats and homogenized in 1 mL of PBS. The tissue homogenate was stored at −80 °C for testing. Virus load was detected with qRT-PCR and expressed as copies/μL for

Fig. 3. Anti-FCV pF(ab')2 helps prevent and treat cats infected with FCV. Prevention group: (A) Compared with the D1 group, the other three groups returned to normal temperatures earlier, and the highest average body temperature was 0.8 to 2 °C higher than that of the B1 and C1 groups; the difference was significant (p < 0.05). (B) The D1 group weights from the fourth day began to decrease, and the average body weight loss rate on the 7th day was 5.4%, which was significantly different from the other three groups (p < 0.05). Treatment group: (C) Compared with the D2 group, the other three groups returned to normal temperatures earlier, and the highest average body temperature was 39.2–40.0 °C. (D) The weights of the D2 group decreased from the fourth day, and the average weight loss rate of the 7th day was 5.3%, which was significantly different from the B2 and C2 groups (p < 0.05). (E and F) After infection with FCV, cats were scored according to clinical symptom scores. On the 7th day after infection, the average scores of the D1 (Prevention group) and D2 (Treatment group) groups were 5.75 and 5.25, respectively, which were significantly different from the other three groups (p < 0.001). n = 4 cats/group; D1, 5 mg/kg NpF(ab')2; D2, 10 mg/kg NpF(ab')2. NS p > 0.05; *** p < 0.001. For Fig. 3B and Fig. 3D, the black dotted line represents the initial weight of the cat.
FCV. The upstream and downstream sequences are 5′-GCAGGTTGGATAAACATGGA-3′ and 5′-CACGAGGCGATTGAGTTGAG-3′.

2.9. Immunohistochemistry test

Tissues were fixed with 20% formalin and embedded in paraffin, and tissue sections were made. The tissue sections were dewaxed, hydrated, washed, and subjected to antigen retrieval using trisodium citrate antigen repair solution (BBI, China). Then, sections were blocked using immunostaining blocking/primary antibody dilution buffer and incubated overnight with anti-FCV antibody (1:500) (VMRD, USA). Sections were incubated with HRP-labeled rabbit anti-feline secondary antibody (Gibco, USA). After DAB (BBI, China) was added for color development, it was counterstained with hematoxylin, and the sections were observed with a microscope (OLYMPUS, Japan).

3. Results

3.1. Evaluation of antibodies

Three 3- to 4-year-old healthy horses were immunized with 4% formaldehyde-inactivated FCV mixed with Freund's complete or incomplete adjuvant intramuscularly 6 times total. Sera were collected serum from three horses. The neutralization titer of IgG in serum was determined by a neutralization assay. The IgG neutralization titer of the 49# horse after the sixth immunization was 1:1230 (Fig. 1A). IgG was extracted by the saturated ammonium sulfate method and digested by pepsin. Further, the purified F(ab′)2 fragment was obtained by protein-G column chromatography. Then, we used SDS-PAGE to assess the integrity of the IgG and pF(ab′)2 fragments (Fig. 1B). The purity of the pF(ab′)2 fragment was over 95% after purification by a protein-G column (Fig. 1C).

3.2. Neutralizing activity of pF(ab′)2

We found that pF(ab′)2 neutralized FCV infection in vitro, and the

![Image](https://via.placeholder.com/150)
half-effective maximal concentration (EC_{50}) of the pF(ab')_{2} fragment was 10.45 μg/mL (Fig. 2A). In an IFA, we demonstrated that pF(ab')_{2} effectively neutralizes FCV infection in vitro and emits green fluorescence (Fig. 2B).

3.3. Equine antibodies neutralized FCV in vivo

We found that when the challenge dose of 10^{7.67} TCID_{50} caused all animals to develop disease. Different doses of pF (ab')_{2} were given to the preventive group the day before the challenge and to the treatment group three days after the challenge. After that, we monitored the rectal temperatures and body weight of the cats for 0–14 days. Cats received pF(ab')_{2}, whether it for prevention or treatment. Their rectal temperatures and weight returned to normal earlier than those control group (NpF(ab')_{2}, red, n = 4) (Fig. 3A–D). We found that both the treatment group and the prevention group (Fig. 3E, F) had significantly (p < 0.001) higher clinical scores on the 7 dpi than the control group than and other groups.

When RT-qPCR was used to detect viral shedding and tissue viral load in the prevention and treatment groups, we found that the cats in the prevention group died when virus shedding was > 7527 copies/μL (Fig. 4A, B). Virus shedding (> 7866 copies/μL) was associated with the deaths of cats in the treatment group (Fig. 4C, D). As expected, anti-FCV pF(ab')_{2} significantly reduced the viral loads in the tracheas, lungs, and spleens in the prevention group (p < 0.05), and anti-FCV pF(ab')_{2} significantly reduced the tracheal and lung viral loads in the treatment group (Fig. 4E, F).

3.4. Anti-FCV pF(ab')_{2} inhibits FCV infection

After we found that pF(ab')_{2} significantly decreased viral load in the trachea, lung and spleen, we further observed that cats injected with pF (ab')_{2}, whether in the preventive or therapeutic groups, had significantly less FCV in these tissues than those injected with NpF(ab')_{2}.
This change suggests that $\text{pF(ab')}_2$ reduces the amount of FCV in different tissues in cats (Fig. 5).

4. Discussion

At present, the treatment of FCV-infected cats is mainly to relieve symptoms but not completely curable in acute-infected cats [16]. Although many scholars have confirmed that compounds can inhibit FCV in vitro, there is no evidence that they can show the same excellent effects in cats.

Neutralizing antibodies provide immediate antiviral protection regardless of the disease state of the cat. Antibodies prepared from horse sera have the advantages of low cost and process maturation and can produce specific antibodies against multiple antigenic determinants. The removal of Fc fragments from IgG is significant in alleviating allergic reactions in animals, making antibodies safer for cats [17]. More importantly, polyclonal F(ab')$_2$ fragments recognize and bind to multiple antigenic determinants of FCV, which may avoid potential antibody escape. Moreover, the F(ab')$_2$ molecular weight is between those of the IgG and Fab fragments. High-molecular-weight IgG has difficulty entering into tissues through blood vessels, which results in delays of antigens neutralizing tissues [18]. Although low-molecular-weight Fab can diffuse into the tissue more easily through the blood vessels, the effect of neutralizing the antigen by the rapid removal via filtration of the kidney is impaired. Second, the half-life of Fab in vivo is less than that of F(ab')$_2$ which results in F(ab')$_2$ being superior to Fab under the same dosage and time [19].

In this study, we demonstrated that the horse anti-FCV serum IgG neutralization titer was as high as 1:1230. The purity of the F(ab')$_2$ fragment was 95.236% after purification with a protein-G column. Next, we found that the F(ab')$_2$ fragment had an E$_{50}$ of 10.45 μg/mL. In the IFA, we found that the specific F(ab')$_2$ fragment of anti-FCV binds to FCV. More importantly, we demonstrate that horse anti-FCV-specific F(ab')$_2$ reduces viral load and virus shedding and has outstanding prophylactic and therapeutic FCV performance.

In vivo, we found that different doses of pF(ab')$_2$ given before or after viral challenge could reduce the clinical symptoms and mortality of infected cats. We found that when the virus shedding amount exceeded 7527 copies/μL or 7866 copies/μL, the animal died. Notably, before we obtained these data, Druet and Hennet et al. found a significant correlation between oral ulceration and FCV load in cats [20], which may also be used as a predictor of survival in animals infected with FCV. When given a certain dose of pF(ab')$_2$, the viral loads in the cats' tracheas, lungs and spleens were significantly decreased in both the preventive and therapeutic groups ($p < 0.05$). These organs produce the feline junctional adhesion molecule A (fJAM-A), a FCV-specific receptor [21]. The reduction in viral load and virus shedding demonstrated that pF(ab')$_2$ inhibited the proliferation of FCV in cats. The immunohistochemistry results also confirmed this phenomenon.

In conclusion, we have successfully prepared an anti-FCV F(ab')$_2$ fragment in vivo and in vitro from equine IgG by immunizing healthy horses with inactivated FCV. Anti-FCV F(ab')$_2$ fragments prepared with horses antibodies reduced viral load in feline trachea, lungs and spleens and prevented FCV in cats in both prophylactic and therapeutic experiments. Thus, horses immunized with inactivated FCV can be used as a source for the development of F(ab')$_2$ fragments. However, the detection of related immune parameters during immunization should be increased. These studies will further confirm that they can be used as a prophylactic and therapeutic agent for diseases caused by FCV.

Author contributions

Zhanding Cui, Dengliang Li, Shushuai Yi, and Guixue Hu conceived and designed the experiments. Zhanding Cui and Dengliang Li performed the experiments. Zhanding Cui and Dengliang Li analyzed the data. Yanbing Guo, Guoying Dong, Han Zhao, Jingting Niu, Ying Zhang, Lili Cao, Guoying Dong and Shushuai Yi contributed reagents/materials/analysis tools. Zhanding Cui and Dengliang Li wrote the paper. Guixue Hu, Yongkun Zhao and Kai Wang requested financial support. All authors read and approved the manuscript.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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