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RESEARCH ARTICLE

Assessment of the Protective Efficacy of a Feline Calicivirus Inactivated Vaccine Using in Vivo FCV CH-JL2 Infection

Yanbing Guo[1, 2], Hongkai Liu[1], Qian Wang[3], Shushuai Yi[1], Jiangting Niu[1], Dengliang Li[1], Zhanding Cui[1], Kai Wang[1]*, Hongze Shao[2]* and Guixue Hu[1]*

[1]College of Veterinary Medicine, Jilin Agricultural University, Changchun, Jilin Province 130118, China
[2]Jilin Institute of Animal Husbandry and Veterinary Science, Changchun, Jilin Province 130062, China
[3]The Third Affiliated Hospital, Changchun University of Chinese Medicine, Changchun, Jilin Province 130117, China
*Corresponding author: wk197811@jlau.edu.cn; guolinyb@163.com; huguixue901103@163.com

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A B S T R A C T

Feline calicivirus (FCV), a highly contagious virus, is one of the major causes of upper respiratory infections in domestic cat populations and wild felines. It is a ubiquitous issue around the world, which constantly demands veterinary attention. Vaccination for FCV has generally been proposed as a cure for its infections. However, the high antigenic variability of this virus hinders the development of efficacious FCV vaccines. In this study, the experimental inactivated vaccine was developed with strain FCV CH-JL2 at $10^{8.00}$ $TCID_{50}$/mL, 1% binary ethylenimine (BEI) was utilized for inactivation at 37°C for 48 h, then was mixed with aluminum hydroxide which was used as an adjuvant. Through regular observation and monitoring, it was confirmed that the kittens in each group had a good mental state and appetite after the vaccination, and their body temperature was normal. Compared with the control group, the antibody levels increased, and no adverse reactions were seen. Its effects were evaluated using the previously constructed animal infection model to measure clinical symptoms after vaccination and FCV challenging. All kittens were challenged with FCV CH-JL2 at 42 days of post vaccination (dpv). The clinical signs of body weight, body temperature, viral shedding, survival rates were monitored daily during 14 days of post challenge (dpc). It has been shown that serum IgG levels and neutralization titer were significantly higher than the commercial inactivated vaccine, and kittens manifested normal clinical signs, good mental status and appetite after vaccination and subsequent viral challenging, the protection rate is 100% (5/5). Collectively, these preliminary data indicate that inactivated vaccine can provide complete protection against infection caused by the challenge FCV CH-JL2 for kittens, and FCV CH-JL2 strain is a promising candidate for developing a safe and effective FCV vaccine in the future.

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INTRODUCTION

FCV belongs to the genus Vesivirus in the Caliciviridae family. It mainly infects kittens under one year old (Binns*et al.*, 2000). FCV infections are commonly associated with oral and upper respiratory tract disease (URTD) in cats (Sykes, 2014). The clinical symptoms mainly manifest as oral ulcer, rhinitis, conjunctivitis, pneumonia and even sudden death(Spiri*et al.*, 2021). Furthermore, this virus causes infections in other felines. Kadoi*et al.* (1997) isolated a strain of FCV from Siberian tigers and African lions in 1992. Tian J *et al.* (2016) isolated the FCV TIG-1 strain from Siberian tiger feces collected in Heilongjiang Province, China, and found it highly virulent in cats. At present, FCV infections has become a worldwide epidemic (Schulz*et al.*, 2011; Afonso*et al.*, 2017; Bordicchia*et al.*, 2021), with a mortality rate as high as 50% (Fumian*et al.*, 2018), which creates threats in both domestic cat populations as well as other felines in the wild, such as tigers and lions (Guo*et al.*, 2018; Pereira*et al.*, 2018; Najera*et al.*, 2021).

Feline calicivirus has only one serotype but the pathogenic significance of individual viral strains is variable in terms of virulence, antigenicity, and heredity

(Brunet*et al.*, 2019). Currently, failure of commercial vaccines in providing full protection is one part of the reason for the pandemic occurred (Rong*et al.*, 2014). In vivo animal infectious models play a crucial role in elucidating infectious pathway of FCV infections. Our previous study had demonstrated the salient pathogenicity and immunogenicity of strain FCV CH-JL2 in stray cats in northeast China (Wang *et al*., 2015; Wang*et al.*, 2017). We, thereby, prepared an inactivated vaccine using this strain expecting to lay a foundation for subsequent investigation on the pathogenic mechanism of FCV and the development of vaccines.

The frequent outbreaks of viral systemic diseases (VSD) in the past decade reflected the poor immunogenicity of the existing commercial vaccines (Bergmann*et al.*, 2019). Therefore, a novel and more effective FCV vaccine for cats based on the emerging variant calicivirus are urgently required. To reach this objective, an experimental inactivated FCV vaccine was developed using the strain FCV CH-JL2. We used an in vivo animal infection model for screening the laboratory-prepared vaccine, and investigated whether vaccination controlled clinical signs and induced antibody levels following FCV infection. Although the results of experimental inactivated vaccine cannot necessarily be directly translated to the clinic, this study will continue to advance our understanding and exploration of the effectiveness of FCV vaccines.

MATERIALS AND METHODS

Animals and ethics statement: Nineteen healthy kittens were originated from normal domestic cats and free from specific pathogens, aged 6-10 weeks and weighing 0.5-0.8 kg were reared in the animal rooms. All kittens had a negative FCV antibody test prior to the study. Each experimental group resided in a separate animal room. Kittens ate food and drank water freely. All experimental kittens were approved and supervised by the Animal Care and Ethics Committee of Jilin Agricultural University (Number 2020 08 05 001).

Virus and vaccine: Feline calicivirus isolate CH-JL2, CH-JL1, CH-JL3 and CH-SH were provided by Jilin Agricultural University and the Institute of Military Veterinary Medicine, Academy of Military Medical Science. The Fel-O-Vax® PCT was commercially purchased (Boehringer Ingelheim Vetmedica, Inc.).

Preparation of the experimental inactivated vaccine: Monolayers of F81 cells were infected with CH-JL2 at 1.0 MOI, and at 24h post infection, the virus liquid was harvested. The fifteenth passage of cell culture-adapted FCV CH-JL2 ($10^{8.00}$ $TCID_{50}$/mL) was chemically inactivated using 1% (v/v) 1mM BEI (EBT SYSTEMS, China) for 48 h at 37°C, shaking at 120 r/min (Wang *et al.*, 2015). The remaining BEI was subsequently neutralized by addition of 20% sodium thiosulfate (Bahnemann, 1990). The effect of inactivation was assessed by the absence of virus growth in F81 cell cultures and by inoculation of cats (n=6), and sterility was checked by inoculation in soybean-casein digest medium and fluid thioglycolate medium (HiMedia, India). The fully inactivated solutions were then mixed thoroughly with 10% (v/v) aluminum hydroxide adjuvant (Rehydragel, SEPPIC, France) and stored at 4°C.

Vaccination and challenge: Thirteen healthy kittens were randomly divided into three groups as follows: experimental inactivated vaccine group (n=5), commercial inactivated vaccine group (n=5), and mock control group (n=3). The injections were conducted subcutaneously twice within a 21-day period, and the injection dose was 1 mL. The kittens in the mock control group were injected with the minimum essential medium (HyClone, China) and adjuvant. Clinical signs were observed and recorded for all kittens during the vaccination campaign. Blood samples were collected, and serum was isolated. Then all kittens were challenged intranasally with 0.5mL FCV CH-JL2($10^{8.97}$ $TCID_{50}$/mL) at 42 dpv according to the infection method established previously (Liu *et al.*, 2018). The clinical signs of body weight, body temperature, viral shedding, survival rates were monitored on a daily basis for 14 dpc. Throat swabs of all kittens were collected, and a nested PCR was performed as described previously to detect FCV infections (Marsilio*et al.*, 2005; Yi *et al.*, 2018).

Antibody assays: Serum FCV IgG levels in kittens were measured using CAT FCV ELISA IgG Kit (Shanghai Enzyme-Linked Biotechnology, China) according to the instructions. Absorbance was read at 450 nm using a microplate reader Model 680 (Bio-Rad).

Neutralization Assay: The neutralization ability of serum antibodies against FCV (CH-JL1, CH-JL2, CH-JL3 and CH-SH) were determined using CPE-determination assays. Briefly, serum samples of heat-inactivated were diluted 2-fold and mixed with an equal volume of 200 $TCID_{50}$ of virus in each well. After 1 h incubation at 37 °C, 100 µL of virus-serum mix was added to the confluent monolayer of F81 cells on a 96-well plate in quadruplicate. The plates were then incubated in a CO_2 incubator at 37°C for 6 days. At the same time, positive and negative controls, virus regression tests, serum toxicity controls, as well as normal cell controls were performed. Neutralization titers were determined by Reed-Muench method.

Histopathological analysis: Lung, trachea, liver and kidney of the kittens were collected 14 days after FCV challenging. All samples were fixed with 4% paraformaldehyde (Solarbio, China) at room temperature. All samples were then sent to Sangon Biotech (Shanghai) Co., Ltd. for histopathological sections.

Statistical analysis: All experiments were repeated at least three times, and data were statistically analyzed using GraphPad 6.0 prism software. Two-tailed *t* tests and ANOVA were utilized to calculate differences, and $P<0.05$ was considered significant.

RESULTS

Post-immunization: Virus inactivation was verified by the absence of viral growth in F81 cell cultures and viral

shedding in inoculated cats. Thus, no typical CPE appeared in F81 cell cultures within 72 h after inoculation, and All kittens had shown normal signs after vaccination, that is, they were all in good mental state, shown normal appetite and behavior, and no eye and nasal secretions (data not shown). The sterile results showed that all samples were free from contamination (data not shown). These results indicated that the virus was completely inactivated, and no live virus was present in the prepared vaccine.

Two kittens in the experimental inactivated vaccine group, one in commercial group and three in the control group developed lumps of a diameter of 2.5 cm at the injection sites. Body temperature of all kittens was measured regularly before and after inoculation, and was shown stable ranging from 37.36 to 37.82°C, no significant change was observed among the three groups (Fig. 1A). Body weight of all kittens was shown increased steadily and the weight gaining rates in the three groups didn't show significantly different between each other, which were 31.71%, 28.57% and 23.81% for groups of experimental, commercial and control group respectively (Fig. 1B).

To detect whether all kittens had FCV shedding before and after inoculation of vaccines, the throat swabs collected periodically were tested by the nested RT-PCR method described above, and the test results were all negative (data not shown).

To measure immune responses induced by the vaccines, peripheral blood of kittens from each group was collected periodically to examine serum IgG levels. Generally, the antibody levels exhibited an obvious enhancement in both the experimental and commercial group, however, the IgG level in the control group remained unchanged. Specifically, the IgG levels in the experimental group was not significantly different from that in commercial group in five-week time after vaccination ($P>0.05$), but they both showed markedly higher compared to that of control group ($P<0.01$). When examined on 42 dpv, the IgG level in kittens of experimental group was significantly different from that in the commercial group ($P<0.05$), also pronouncedly higher than that in mock control group ($P<0.01$). The IgG level in cats of the commercial group was higher than that of mock control group ($P<0.01$) (Fig. 1C).

Previous test results showed that the immune response level was relatively highest at 2 weeks after immunization (Wang *et al.*, 2015), so we detected the serum neutralization titer of each group. The results showed that the average neutralizing antibody titer of the experimental inactivated vaccine group was significantly higher than those in the mock control group, and the same as that of the commercial inactivated vaccine group. However, compared with the commercial group, the neutralization titer of the experimental group for non-homologous virus strains (CH-JL3) and homologous virus strains (CH-JL2) was higher, and the difference was significant ($P<0.05$) (Fig. 1D).

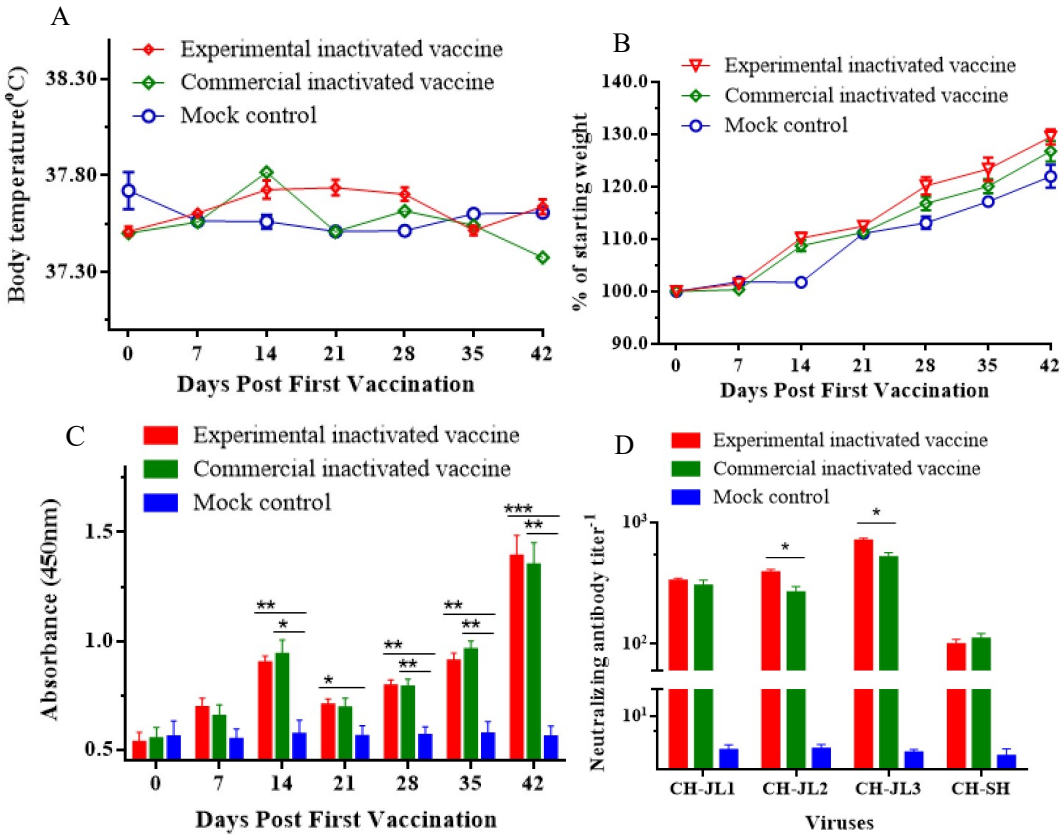

Fig. 1: Post-immunity responses of kittens. (A) Body temperature (°C). (B) Increase rate of Body weight (%). (C) IgG levels in serum detected by ELISA. (D) Neutralization titers induced in the different immunization groups. The data is shown as the means ± S.E.M (n=3-5) and was analyzed using a one-way ANOVA (*P<0.05; **P<0.01; ***P<0.001).

Protective effects of vaccines against FCV CH-JL2 challenge: The cats were challenged with FCV CH-JL2 at 42 days after the first vaccination. Then clinical signs were recorded daily after challenged with FCV. Cats in the control group typically showed FCV infectious symptoms, such as oral nasal ulcer, purulent secretion of the canthus, depressed spirit and dyspnea. One kitten died on 10 dpc, and the other on 11 dpc. However, all the cats in both experimental and commercial group remained in good health with no death (Fig. 2A).

Body weight was monitored on a daily basis (Fig. 2B). Compared to the initial body weight, the average body weight of the control group reached its lowest on 10 dpc after the death of the kittens, and the body weight of the surviving kitten remained stable. During this period, the average body weight of kittens in the experimental and commercial group increased by 19.74% and 19.34% respectively.

Changes in body temperature was observed in Fig. 2C. The average body temperature of cats in control group increased from 2 dpc onwards. Except for some dead, the body temperatures of remaining kittens in this group were abnormally high, up to 40.3°C. However, body temperature of kittens in the experimental and commercial group only slightly increased by an average of 0.3°C on 2 dpc-3 dpc, and then dropped to normal values in the remaining period. No significant difference was observed between these two groups (P>0.05).

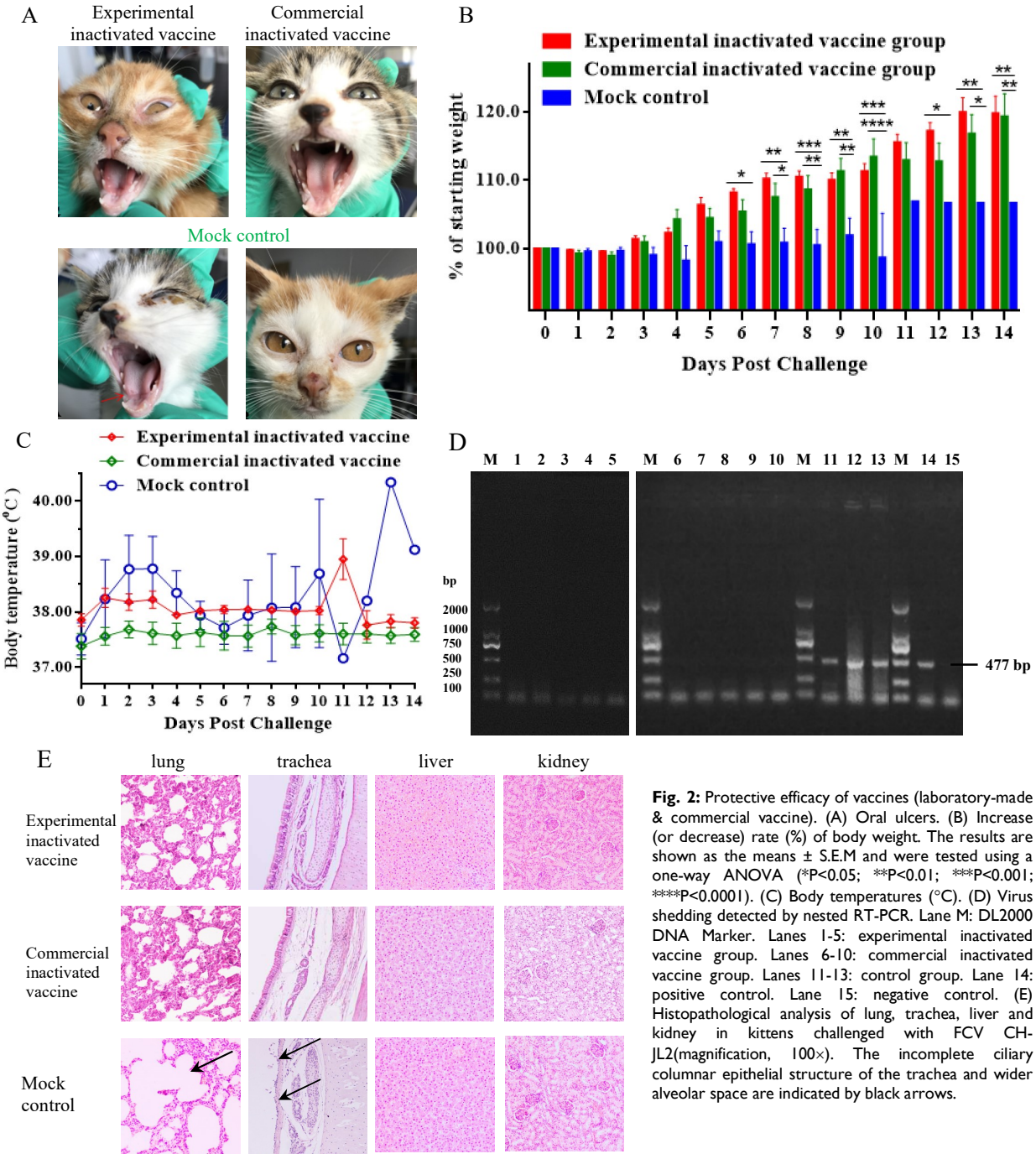

Fig. 2: Protective efficacy of vaccines (laboratory-made & commercial vaccine). (A) Oral ulcers. (B) Increase (or decrease) rate (%) of body weight. The results are shown as the means ± S.E.M and were tested using a one-way ANOVA (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001). (C) Body temperatures (°C). (D) Virus shedding detected by nested RT-PCR. Lane M: DL2000 DNA Marker. Lanes 1-5: experimental inactivated vaccine group. Lanes 6-10: commercial inactivated vaccine group. Lanes 11-13: control group. Lane 14: positive control. Lane 15: negative control. (E) Histopathological analysis of lung, trachea, liver and kidney in kittens challenged with FCV CH-JL2(magnification, 100×). The incomplete ciliary columnar epithelial structure of the trachea and wider alveolar space are indicated by black arrows.

Table 1: Virus shedding after challenged with FCV CH-JL2 by nested RT-PCR

Group	3 d	4 d	5 d	6 d	7 d	8 d	9 d	10 d	11 d	12 d	13 d	14 d
Experimental inactivated vaccine	0/5	3/5	4/5	2/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Commercial inactivated vaccine	1/5	2/5	4/5	3/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Mock control	0/3	2/3	3/3	3/3	3/3	3/3	3/3	2/2	1/1	1/1	1/1	1/1

In the experimental inactivated vaccine group, three kittens were detected positive of FCV CH-JL2 on 4 dpc, and increased to four on 5 dpc, reduced to two on 6 dpc, then all kittens showed absent of the virus after 7 dpc.In the commercial inactivated vaccine group, one kitten was detected positive for the virus on 3 dpc, increased to two on 4 dpc, and four on 5 dpc, then decreased to three on 6 dpc, and one on 7 dpc, then all showed absent of the virus after 8 dpc.In the control group, two kittens were detected positive on 4 dpc, then all showed positive for this virus after 3 dpc. Two kittens died on d 10 and 11, respectively.

Throat swabs of kittens in each group were collected daily and virus shedding was detected by nested RT-PCR. The virus was detected in four out of five cats in each of the experimental and commercial groups on 5 dpc. All kittens in the experimental group were negative for the presence of the virus after 7 dpc; and in the commercial group after 8 dpc. All kittens in the control group constantly showed positive presence of virus after 5 dpc (Table 1). Through 8 dpc, one representative image from the agarose gel electrophoresis of nested RT-PCR products is shown (Fig. 2D).

No visible lesion in other organs was observed in kittens of both experimental and commercial group by autopsy. However, mucus of trachea turned yellow-white and a small area of pulmonary lobe was congested in kittens of control group. Histopathological analysis revealed no significant difference between the kittens in experimental and commercial group. Cats in control group showed incomplete ciliary columnar epithelial structure of the trachea, wider alveolar space and a small amount of lymphocyte and macrophage infiltration in the lungs, while no changes were observed in other organs (Fig. 2E).

DISCUSSION

In the present study, we developed an FCV inactivated vaccine and used a cat infection model with Feline calicivirus strain CH-JL2 to evaluate efficacy of the vaccine. Currently, vaccines are the primary options for the prevention of FCV infections. Vaccination has reduced the incidences of FCV infections, however, high variability of FCV has resulted in frequent immune failure. More vaccines are being developed to address this problem (Poulet*et al.*, 2005; Addie*et al.*, 2008). We isolated three FCV strains in Jilin Province, China (Zhao*et al.*, 2017), and our previous research showed that strain FCV CH-JL2 could be used as a candidate for the development of FCV vaccine in terms of its immunogenicity (Wang *et al.*, 2015).

Kittens are at the most susceptible age for infection FCV (Binns*et al.*, 2000). Published studies have also shown that kittens under 12 months are highly vulnerable to co-infection by FCV with other pathogens, such as feline herpesvirus-1 (Zicola*et al.*, 2009), Mycoplasma felis(Berger*et al.*, 2015) and feline parvovirus (Lappin*et al.*, 2002; Dall'Ara*et al.*, 2019), which makes therapies more complex. So, six to 10 weeks old kittens were selected for this study. According to the previous successful challenging cases using FCV, to simulate a natural infection, a unified nasal challenge was performed to reduce system error (Liu *et al.*, 2018). The virus incubation period was 1 to 2 days after infection; it took 5-7 days that virus shedding reached a peak; within 8 to 10 days, some kittens died; the course of infection could last 7 to 11; and the survival kittens entered into the transition period after 13 days of infection. Our strain FCV CH-JL2 used in this study, isolated from infected kittens, could not be detected in tissues except for lung, and could only cause oral ulcers and upper respiratory tract inflammation. This suggests that strain FCV CH-JL2, similar to F9 (Tian*et al.*, 2020), was mild in virulence and unable to cause systemic infections, which are optimal for vaccine investigation.

BEI is an alkylating agent, which is widely used because of its easy storage, low toxicity and cost (Kai and Chi, 2008; Geldhof*et al.*, 2012; Adi*et al.*, 2019). This inactivated vaccine could mainly provoke humoral immunity in the body. In this study, IgG levels and neutralization titers were significantly increased after immunization compared with commercial vaccines, suggesting that the prepared inactivated vaccine had a partial cross-protection response. Considering all these factors, we believe that the FCV CH-JL2 strain has promise to be developed as a safe and efficacious vaccine against viral disease. Information from this study first showed the potential for experimental inactivated vaccine development for FCV CH-JL2 strains. Future research should focus on how to improve the ability of the vaccine to activate the innate immune system and against the antigenic variants of the field viruses.

Despite promising data obtained in this study, there were a few considerations. Firstly, kitten under one year old generally lacks of autoimmunity and are susceptible to their surroundings, especially temperatures and feed, which could result in their unsteady immune status, which may further affect the accuracy and consistency of final outcomes. Secondly, the parental immune background of the kittens used in this study is unknown, and could have had an impact on the immunization of FCV vaccination. Finally, different strains of FCV were with different levels of virulence. In our research, we only challenged the CH-JL2 strain to assess immune protection. Whether the prepared inactivated vaccine can induce protective effect against other strains of FCV needs to be further researched.

Conclusions: In this study, we developed an experimental inactivated FCV vaccine using the CH-JL2 strain of FCV with significantly higher neutralizing antibody titers than commercial vaccine, which controlled the clinical symptoms and induced antibody levels after FCV infection. The inactivated vaccine developed based on the CH-JL2 strain has a good protective effect against FCV infection, and can be used as a candidate strain for the development of vaccine, which lays a foundation for the development of clinical vaccine against FCV in the future

and the effective prevention of the occurrence and transmission of the disease. In addition, good management practices for animal welfare during the experimental period is a warrantee for reliable outcomes.

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Authors contribution: YBG, HKL and GXH: conceived and designed the experiments. HKL, QW and SSY: performed the experiments. YBG, HKL and JTN: analyzed the data. DLL, ZDC and KW: contributed reagents/materials/ analysis tools. YBG, QW and GXH: wrote the paper. YBG and HZS: revise the paper.

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