

Antigenic and Plaque Variations of Serotype II Feline Infectious Peritonitis Coronaviruses

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ABSTRACT. Three feline coronavirus (FCoV) isolates KUK-H, M91-266, and M91-267 were examined to elucidate their biological and antigenic properties as well as disease potential in cats. Immune stainings of virus-infected cells by using FCoV type-specific monoclonal antibodies indicated that their antigenic specificity was serotype II. However, antigenic variations among these serotype II FCoVs were detected by neutralization assay with hyperimmune antisera against FCoVs and canine coronaviruses, and with experimentally infected cat sera; there were two subtypes in serotype II FCoVs. The isolates efficiently grew in fcwf-4 cell culture showing lytic CPE enough to form distinct plaques; when measured 48 hr after infection, plaque sizes of both M91-266 and M91-267 were approximately 1 mm in diameter, and a mixture of small (less than 1mm in diameter) and large (approximately 3 mm in diameter) plaques were produced in the case of KUK-H. Strains KUK-H, M91-266 and M91-267 produced feline infectious peritonitis (FIP) in 50%, 67% and 89% of experimentally inoculated kittens, respectively. Furthermore, 80% of the kittens inoculated with the small plaque former of KUK-H developed FIP accompanied by more prominent clinical signs as well as pathological changes when compared with 28.6% of kittens inoculated with the large plaque former. These results suggest that serotype II FIPVs producing smaller size of plaques are more virulent than those producing larger size of plaques. — **KEY WORDS:** coronavirus, feline, feline infectious peritonitis, plaque, virulence.

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According to the recent concept about feline coronaviruses (FCoV) [21], there are two biological types of coronavirus in cats, that is, an enteritis-inducing feline enteric coronavirus (FECV) and a systemic pathogen feline infectious peritonitis virus (FIPV) which also causes enteritis locally. In respect of genetic and antigenic properties, however, they are virtually the same virus so that the generic name “FCoV” is recently adopted to encompass both two viruses. In other words, one could identify an isolate as FIPV or FECV, regardless of clinical conditions of the cat from which it was isolated, only after experimental determination of its disease-causing potential in cats. FECV is epizootiologically the predominant FCoV in the field, and it has been tentatively concluded that FIPV is a mutant of FECV, though the exact mutation mechanism has yet to be clarified [4, 21, 23, 29].

FCoVs are divided into two distinct serotypes I and II by neutralization specificities induced by a spike protein [5, 10, 11, 19]. Serotype I FCoV isolates have been numerically superior [22] and predominantly distributed among cats [9]. These differences in antigenic properties are generally consistent with viral growth ability *in vitro* [2, 22]; serotype II FCoVs propagate more readily than serotype I FCoVs in feline cell cultures such as *Felis catus* whole fetus-4 (fcwf-4) [13] and Crandell feline kidney (CRFK) cells. In addition, serotype II FCoVs are more efficiently neutralized by antiserum against canine coronavirus (CCV), indicating their closer antigenic relatedness with CCV. Recent molecular studies [6, 20, 29] suggested a possibility that

serotype II FCoV has arisen by recombination between serotype I FCoV and CCV. In the present paper, three serotype II FCoV isolates from clinical FIP cases were examined in respects of antigenic property and *in vivo* pathogenic virulence especially in relation to plaque characteristics. Discussion was also made regarding relationships between plaque properties and virulence of coronaviruses in animals.

MATERIALS AND METHODS

Viruses and cell culture: KUK-H strain was isolated in 1987 by using CRFK cells (ATCC CCL94), and both M91-266 and M91-267 strains were isolated in 1991 by using fcwf-4 cells. All derived from the spleen samples taken at the postmortem examination of effusive form FIP field cases. Stock viruses were prepared after further several passages in fcwf-4 cell culture. Strain C3663, a local isolate from an effusive form FIP case, was used as a reference serotype I FCoV. The cells were cultured as described previously [18].

Plaque assay: The stock viruses were characterized by plaque assay, and then the virus was plaque-purified for further experiments. After serial tenfold virus dilutions, 0.2 ml of each dilution was inoculated onto fcwf-4 cell monolayer in 60-mm plastic plates. The plates were incubated at 37°C for 1 hr, and 4 ml of an overlay medium was added. The plates were further incubated at 37°C for 2 or 3 days and then stained with either an overlay medium

containing 0.01% neutral red or a mixture of 10% formalin and 0.5% crystal violet solution.

Serology: Identification and serotyping of the isolates were performed by either indirect immunoperoxidase or immunofluorescence staining using monoclonal antibodies (MAb) against FCoV [10, 11]. Antigenic analysis was performed by plaque-reduction neutralization assay using hyperimmune antisera as well as experimentally infected cat sera, and the method was as described previously [18]. Briefly, each of twofold serum dilutions was mixed with 100 PFU of virus, and incubated at 37°C for 1 hr. Then 0.2 ml of each of the mixture was inoculated onto fcwf-4 cell monolayer in the plate. The plates were treated by the same manner as the plaque assay afterwards. The titer was expressed as the reciprocal of the highest serum dilution showing 75% plaque count reduction or more.

Hyperimmune antisera against KUK-H and M91-266 were made using specific-pathogen-free guinea-pigs and mice, respectively. Hyperimmune rabbit sera against CCV strains 5821 and 1-71 were obtained from Kyoto-Biken Laboratories and the University of Tokyo, respectively, and their specificity has been described previously [18, 27]. Serum samples of experimentally infected cats (Table 2) were collected just before euthanasia.

Animal experiments: A total of 33 conventional kittens were used and a litter of kittens was impartially divided into each experimental group. They were about two-month-old and negative (<1:10) for neutralization antibody against the inoculum. They were also negative for feline leukemia virus antigen in blood and feline immunodeficiency virus antibody throughout each experiment. They were observed daily for clinical signs and determined weekly for blood counts for two or three months after intraperitoneal inoculation of virus. Inoculum dose for each experiment was shown in Tables 2 and 3. When the animal in a critical condition, they were examined pathologically after euthanasia.

RESULTS

Antigenic properties: The fcwf-4 cells infected with each isolate were positively stained with both a MAb reagent (N+M+S: a mixture of MAbs against each nucleocapsid, integral membrane, and spike proteins of FCoV) and a MAb (5-6-2) specific to serotype II FCoV but not with a MAb (U1-1) specific to serotype I FIPV, indicating that they are serotype II FCOVs.

When neutralization antigenicities of the isolates were examined with polyclonal hyperimmune antisera, M91-266 was not neutralized by the sera against both KUK-H and CCVs so efficiently as KUK-H and M91-267 (Table 1). This property was also observed when the experimentally infected cat sera were used; however, M91-267 was neutralized by the cat sera infected with M91-266 as efficiently as homologous M91-266 (Table 1).

Plaque characterizations: Plaques produced in fcwf-4 cells by the isolates were readily observed 48 hr after

Table 1. Neutralization antigenicities of FCOVs determined with hyperimmune and experimentally infected cat sera

Sera used in the assay	Virus strains		
	KUK-H	M91-266	M91-267
<i>Hyperimmune antisera</i>			
Anti-KUK-H	32	<2	32
Anti-M91-266	160	640	160
Anti-CCV 5821	320	20	320
Anti-CCV 1-71	640	20	320
<i>KUK-H infected cat sera</i>			
Cat #I	1,000	<100	4,000
Cat #II	1,000	<100	4,000
Cat #III	4,000	100	8,000
<i>M91-266 infected cat sera</i>			
Cat #1	4,000	64,000	32,000
Cat #2	1,000	32,000	32,000
Cat #3	8,000	32,000	64,000
Cat #8	4,000	32,000	64,000
Cat #9	400	4,000	3,200
Cat #10	200	4,000	3,200
<i>M91-267 infected cat sera</i>			
Cat #11	16,000	800	32,000
Cat #13	4,000	800	8,000
Cat #14	2,000	800	8,000
Cat #16	2,000	100	16,000
Cat #17	2,000	100	8,000
Cat #18	2,000	200	8,000
Cat #19	2,000	400	16,000

infection. They were approximately 1 mm in diameter for both M91-266 and M91-267, and KUK-H showed a heterogeneous plaque size distribution consisting of small (less than 1 mm in diameter) and large (about 3 mm in diameter) plaques (Fig. 1). Although small (KUK-H/S) and large (KUK-H/L) plaque formers were purified from the parental stock (Fig. 2), no significant antigenic difference was observed between them and they grew in fcwf-4 cell cultures showing similar CPE. Their infective titers also reached 10^5 to 10^6 PFU/ml 24 hr after inoculation at a multiplicity of infection of 0.005, but KUK-H/L tended to grow faster and less cell-associated than KUK-H/S.

FIP inducing virulence: On an average, 50%, 67% and 89% of the kittens inoculated with KUK-H, M91-266 and M91-267, respectively, were diagnosed as either effusive or non-effusive form FIP between the post inoculation day 9 and 72, indicating that the isolates are FIPV of FCOVs (Table 2). Serum neutralization titers were converted to 1:1,000-1:64,000 in all animals regardless of their clinical conditions. With the exception of a kitten which developed FIP after inoculation of KUK-H, viruses were recovered from either spleen or liver, or both of all animals regardless of clinical manifestation. In general, animals inoculated with less virus doses tended to develop more fulminating FIP within a shorter period, and M91-267 appeared to be most virulent among them.

Virulence of small (KUK-H/S) and large (KUK-H/L)

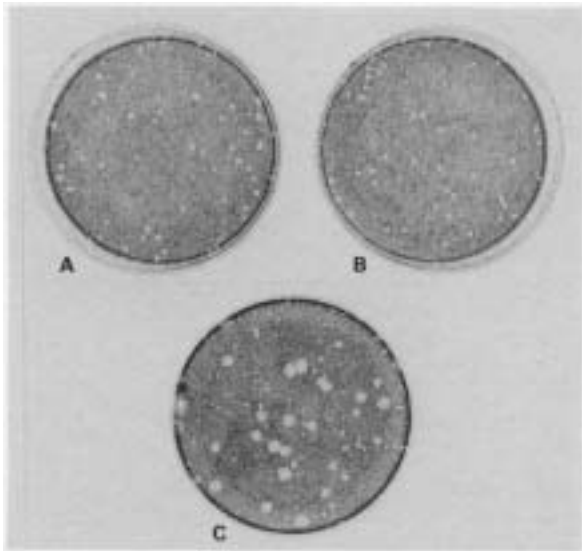


Fig. 1. Plaques produced 48 hr after infection in the fcwf-4 cells by M91-266 (A), M91-267 (B) and KUK-H (C) strains.

plaque formers of KUK-H strain: A comparison was made of FIP symptoms induced when KUK-H/S and KUK-H/L were inoculated into 12 kittens intraperitoneally. As shown in Table 3 and Figs. 3 and 4, four out of five kittens (80%) inoculated with KUK-H/S developed FIP and three were effusive and the other was non-effusive form of FIP. Meanwhile, two out of seven kittens (28.6%) inoculated with KUK-H/L developed effusive and non-effusive forms of FIP, respectively. Serum neutralization titers of the kittens inoculated with KUK-H/S were between 1:2,000 and 1:256,000, and with KUK-H/L were between 1:100 and 1:32,000. Small plaque forming viruses were recovered from both spleen and liver of one kitten No. S-7 which developed effusive form of FIP after inoculation with KUK-H/S on the post inoculation day 19. However, no virus was recovered from the other kittens regardless of clinical manifestation.

The kittens inoculated with KUK-H/S manifested more severe clinical signs than the kittens inoculated with KUK-H/L, but diarrhea was more frequently observed in the kittens inoculated with KUK-H/L (Fig. 3).

Histopathological changes were observed mainly in liver, spleen, kidney, lung, small intestine, mesenteric lymph nodes, thyroidea, and urinary bladder, and were summarized in Fig. 4 referring to the typical changes produced in FIP cats. The extent of damage of each tissue was severer in the kittens inoculated with KUK-H/S than KUK-H/L.

DISCUSSION

Results obtained in the present study may provide new information on considering properties of serotype II FCoV; one is a heterogeneous nature of neutralizing antigenicity and another is the plaque size which could be an *in vitro*

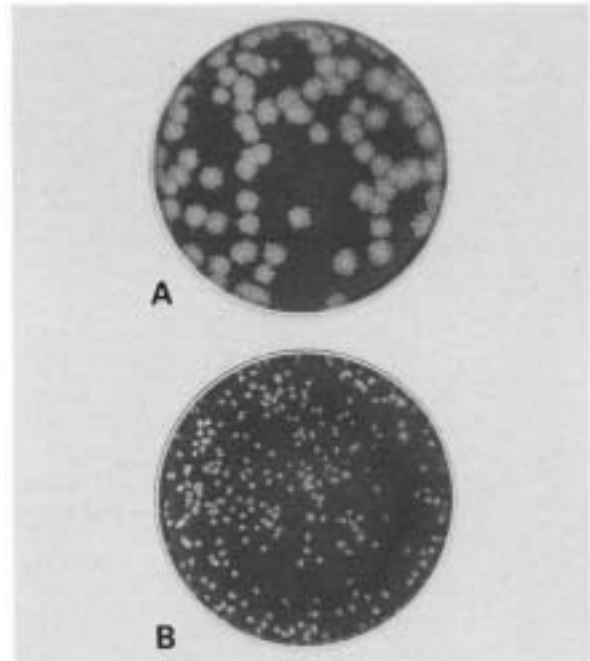


Fig. 2. Plaques produced 72 hr after infection in the fcwf-4 cells by KUK-H/L (A) and KUK-H/S (B) plaque formers.

marker for FIP-inducing virulence.

At present, the following perspectives concerning FCoV evolution have been proposed: FIPVs arise in some way from most probably indigenous FECV lineage, and serotype II FCoVs are generated by the recombination between serotype I FCoV and CCV [12, 29]. However, it is suspected that the recombination events between serotype I FCoV and CCV may not always occur to an equal extent and at the same genomic site of the genome. Thus, there must be variations in pathogenic and antigenic properties among the resultant serotype II FCoVs. Indeed, serotype II FIPV 79-1146 strain is considered to be typical: the S gene and an adjacent downstream are characteristic to those of other serotype II FCoV and CCV, but the M gene and gene 4 are those of serotype I FCoVs [29]. Another example is a CCV isolate reported in England [15]. Differing from a general consideration that CCVs are non-pathogenic against cats [1, 17, 24], the CCV isolate induced FIP in cats [15]. It is more pertinent, therefore, to regard it as FCoV-like CCV generated by some genetic event between genuine CCV and serotype I or serotype II FCoV. If these considerations are generally applicable to serotype II FCoVs and CCVs in nature, it is not astonishing to have obtained the result that serological reactivities of M91-266 is different from those of KUK-H and M91-267 (Table 1), suggesting that there are two neutralization subtypes among serotype II FCoVs. Antigenic relationships were more complicated when the post-infected cat sera were applied in the assay where one way cross-neutralization between M91-266 and M91-267 was observed (Table 1).

Previous reports on the plaque characteristic in relation

Table 2. Feline infectious peritonitis (FIP)-inducing virulence of FCoV isolates by intraperitoneal inoculation

FCoV strains	Inoculation dose (PFU)	Animals inoculated	Animals developed FIP
			(the average post inoculation days)
KUK-H	5.3×10^4	3	2 (14.5)
	1.6×10^6	3	1 (20)
M91-266	1.8×10^5	3	3 (27.3)
	1.3×10^7	3	1 ^{a)} (72)
M91-267	3.8×10^5	3	3 (9.3)
	5.3×10^6	6	5 ^{b)} (13)

a) Non-effusive form FIP.
 b) Four out of five were non-effusive form FIP.

Table 3. Feline infectious peritonitis (FIP)-inducing virulence of small (KUK-H/S) and large (KUK-H/L) plaque formers

Inoculation dose (PFU)	KUK-H/S		KUK-H/L	
	Animals		Animals	
	:inoculated	:developed FIP	:inoculated	:developed FIP
2.0×10^4	2	2	2	0
2.3×10^4	3	2 ^{a)}		
3.5×10^4			3	1 ^{b)}
5.0×10^4			2	1
Total	5	4	7	2

a) One out of two was non-effusive form FIP. b) Non-effusive form FIP.

KUK-H/S inoculated animals

Inoculation dose (PFU)	Clinical signs ^{a)}	Diagnosis ^{b)}				
			Depression	Anorexia	Vomiting	Diarrhea
2.0×10^4	No. S-9	E/FIP				
	No. S-10	E/FIP				
2.3×10^4	No. S-6					
	No. S-7	E/FIP				
	No. S-8	N/FIP				

KUK-H/L inoculated animals

2.0×10^4	No. L-8	
	No. L-9	
3.5×10^4	No. L-1	
	No. L-2	N/FIP
	No. L-3	
5.0×10^4	No. L-4	
	No. L-5	E/FIP

Fig. 3. Clinical signs of the kittens inoculated with either KUK-H/S or KUK-H/L. a) Severity ++, +, +/-, -. b) The animals were diagnosed based on the histopathological findings summarized in Fig. 4. E/FIP; effusive form FIP, N/FIP; non-effusive form FIP.

to both serotypes and disease potential [2, 3, 16, 28] are somewhat conflict with each other as well as with the present results. Highly virulent FIPV strains NOR15 and 79-1146 produced larger homogenous plaques in comparison to FECV 79-1683 and CCV 1-71 strains which produced a heterogenous population of plaque sizes [16, 28]. Plaque size varied considerably between strains in another study; both highly virulent FIPV strain 79-1146

and non-virulent FECV strain 79-1683 produced larger plaques than the other highly virulent FIPV strains UCD1 and Black (low passaged) [2]. Such inconsistent description of the plaque size of the same strains may result from the culture conditions applied in each experiment [2, 16, 28]. For example, large plaques tend to be produced in fcwf-4 cells [2] when compared with CRFK cells [16, 28]. Furthermore, overlay media considerably influences plaque

KUK-H/S inoculated animals						KUK-H/L inoculated animals						
Tissues	2.0 x 10 ⁴ PFU		2.3 x 10 ⁴ PFU			2.0 x 10 ⁴ PFU		3.5 x 10 ⁴ PFU			5.0 x 10 ⁴ PFU	
	No.S-9	No.S-10	No.S-6	No.S-7	No.S-8	No.L-8	No.L-9	No.L-1	No.L-2	No.L-3	No.L-4	No.L-5
Liver												
Spleen												
Kidney												
Lung												
Cardiac muscle												
Pancreas												
Stomach												
Duodenum												
Jejunum/Ileum												
Large intestine												
Mesenteric l.n.												
Cerebrum												
Cerebellum												
Adrenal gland												
Thyroid gland												
Bone marrow												
Hypophysis												
Bladder												
Genitalia												
Diagnosis	E/FIP ^{b)}	E/FIP		E/FIP	N/FIP	ND ^{c)}		ND				E/FIP

Fig. 4. Histopathological changes of the kittens inoculated with either KUK-H/S or KUK-H/L. a) The extent of severity is expressed as -, +/-, +, ++, and +++ as shown in Fig. 3. b) E/EIP; effusive form FIP, N/FIP; non-effusive form FIP. c) ND; not determined.

formation [26]. From these limited reports under the different conditions, it was difficult to know a correlation between plaque sizes, disease potential, and FIP-inducing virulence of FCoV. Furthermore, serotype I FIPVs are generally difficult to grow in cell cultures [21, 22], presumably resulting in no plaque formation irrespective of their FIP-inducing virulence. Although it was described that the plaques of FECV 79-1683 were heterogenous in size despite three cycles of plaque purification [16], the small plaque former KUK-H/S and the large plaque former KUK-H/L were successfully purified from the same parental stock in the present study. Of particular interest is that different FIP-inducing virulences of KUK-H/S and KUK-H/L were observed. As shown in Table 3, KUK-H/S produced FIP in cats at a high rate. On the contrary, FIPV KUK-H/L caused diarrhea more frequently but to less extent FIP in the cats, suggesting that KUK-H/L is FECV-like FIPV.

The present results suggest that small plaque-forming serotype II FIPVs are more virulent with regard to FIP-inducing capacity than the large plaque-forming one, though it needs further examinations using more FIPV strains under the same experimental conditions. If this is generally found in serotype II FIPVs, however, the present hypothesis is not in agreement with general understanding about the relationship between on plaque sizes, attenuation and virulence of animal viruses including other coronavirus species [7, 8, 14, 26, 30]. Small plaque formers tend to be less virulent than their large plaque counterparts, though there are some exceptions [26]. Therefore, it is worth attempting the following additional genetic and biological

experiments in connection with this subject: (1) to examine the ORF7b transcription unit of FCoV genome encoding a nonvirion secretory glycoprotein (gp7b), and (2) to examine growth in feline macrophages *in vitro*, because it has been described that deletion of ORF7b appears to be correlated with reduced virulence of FCoVs [6], and avirulent FCoVs infect and replicate in the cells far less efficiently than the virulent FCoVs [25], respectively.

Finally, we must consider that immune-mediated pathogenic mechanisms proposed for developing clinical FIP in cats [21, 22] may complicate assessment of viral virulence more. This mechanism is possibly related to the observation that the animals inoculated with higher virus doses did not always induce clinical FIP at a higher frequency (Table 2). Immune system of the inoculated animals may have influenced the disease course, though the exact reason is not clear at present.

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