

Plaque Assay for Canine Coronavirus in CRFK Cells

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Canine coronavirus (CCV) has been identified as one of the causative agents of viral enteritides in members of the *Canidae* and has distributed worldwide [1, 2, 5, 6, 8, 12, 14, 16]. Primary dog cell cultures [1, 2, 11, 16] and several cell lines [3, 6, 7] have been widely used for infectivity assay of CCV. Except that one cell line, FC cells [15], was applied for plaque assay of a CCV strain, little is known about plaquing ability of other CCV strains. The present note describes the plaque assay of three strains of CCV in CRFK cells which were established from domestic feline kidney by Crandell et al. [4], and were obtained from Dr. Weaver, Naval Bioscience Lab., Univ. California, Calif.

CCV strain 1-71 [2] was obtained from American Type Culture Collection (Rockville, Md.). Toda [16] and 5821 strains were supplied by Dr. Kojima (Tanabe Seiyaku Co., Ltd., Osaka, Japan), and Dr. Ajiki (Kyoto-Biken Lab., Kyoto, Japan), respectively. All the strains were passaged in CRFK cells.

CRFK cells were grown at 37°C in Eagle's minimum essential medium (MEM) (Gibco, USA) supplemented with 8% heat-inactivated fetal calf serum (FCS), 10% tryptose phosphate broth (Difco, USA), and antibiotics. The concentration of FCS was reduced to 1% in a maintenance medium (MM) for virus propagation.

Anti-serum against each strain was prepared in rabbits. Viral antigens purified by the method of Makino *et al.* [9, 10] were mixed with Freund's complete adjuvant, and injected into foot pad of rabbits. Three weeks later each rabbit received a further injection with the same manner, and sera were obtained one week after the booster inoculation.

For plaque assay, cell monolayers were prepared in 60 mm petri dishes by seeding cells suspended in 5 ml of growth medium (GM) and

by incubating at 37°C in a humidified atmosphere containing 5% CO₂. After virus inoculation cell sheet was overlaid with agar medium (AM) consisted of MM containing 1% Bacto-agar (Difco, USA). Strain 1-71 was used to determine experimental conditions for plaque assay.

As shown in Table 1, 0.11% sodium bicarbonate for GM and 0.22% for AM gave clear plaques and produced the maximum number of plaques. There was no significant difference of plaque formation by seeding of 0.5 to 2×10⁶ cells per plate.

Kinetics of virus adsorption onto CRFK cells is shown in Table 2. In unwashed cultures after the adsorption, the number of plaques increased and reached maximum level within 60 min of incubation. In cultures washed with Dulbecco's phosphate-buffered saline (PBS), the number of plaques reached the same level as unwashed ones when the adsorption time was lengthened to 90 min. There was no difference in virus adsorption, when MEM or PBS was used as virus diluent. Addition of 1% and 5% of FCS or 0.1% bovine serum albumin did not affect virus adsorption.

Table 3 shows the effect of the incubation time after virus inoculation. Cytopathic effect was first observed after 24 to 36 hr of incubation, and clear plaques were visible without staining with neutral red after 36 to 48 hr. After 72 hr of incubation plaques increased in size but not in numbers. After 96 hr of incubation the whole cells became dead and plaques disappeared.

The plaque number was shown to be directly proportional to the virus concentration, indicating that one infectious particle was sufficient to produce one plaque.

Plaques produced by three CCV strains are shown in Fig. 1. Strain 1-71 formed the largest plaques and strain Toda formed the smallest ones. Plaque formation of each strain was equally inhibited by 50% plaque reduction method with the specific anti-sera against all strains.

The following method was based on the results hitherto presented: CRFK cells were seeded in

Table 1. Determination of proper bicarbonate concentration on plaque formation

Concentration of NaHCO ₃ in (%)		Plaques	
GM ^{a)}	AM ^{b)}	Number ^{c)}	Appearance
0.11	0.11	71.4± 6.5	Less clear
0.11	0.22	104.2±10.5	Clear
0.22	0.11	39.8± 9.8	Unclear
0.22	0.22	83.6± 9.7	Clear

- a) Growth medium at cell seeding.
 b) Agar medium overlaid after virus adsorption.
 c) Mean plaque numbers of five dishes±standard deviation.

Table 3. Effect of incubation time on plaque formation

Incubation ^{a)} (hr)	Plaques	
	Number ^{b)}	Size ^{c)} (mm)
48	86.0±13.7	0.5–2.0 (1.3)
72	89.6±11.4	0.7–2.9 (1.5)
96	— ^{d)}	— ^{d)}

- a) Incubation at 37°C before staining with neutral red.
 b) Mean plaque numbers of five dishes ± standard deviation.
 c) Range of plaque diameter and average diameter of 20 plaques in parenthesis.
 d) Plaques were not visible by cell damage.

60 mm dishes with 5 ml of GM containing 0.11% sodium bicarbonate and incubated at 37°C in a humidified atmosphere containing 5% CO₂ until monolayers were formed. The cultures were washed once with PBS, and inoculated with 0.2 ml of virus dilution in MEM containing 1% FCS. After virus adsorption at 37°C for 90 min, the cultures were covered with AM containing 0.22% sodium bicarbonate without washing, incubated at 37°C for two days and stained by 0.01% neutral red in PBS containing 1% Bacto-agar. After incubation at 37°C for 6 to 8 hr, the plaque number was counted and the infectious titer was expressed in plaque forming units. It has been reported that plaque formation of some coronaviruses was enhanced by trypsin in AM [13], but there was no significant effect of trypsin on the CCV-CRFK cell system.

In the present study, CRFK cells were satisfactory for plaque formation of CCV. The method was simple, reproducible for virus infectivity assay, and applicable to neutralization tests.

Table 2. Kinetics of virus adsorption on CRFK cells at 37°C

Adsorption (min)	Plaque number	
	Unwashed	Washed ^{a)}
0	24.8 ^{b)}	4.2
30	59.0	40.4
60	84.0	58.2
90	79.0	85.4
120	78.0	80.8

- a) Three times with PBS after virus adsorption.
 b) Average of five dishes.

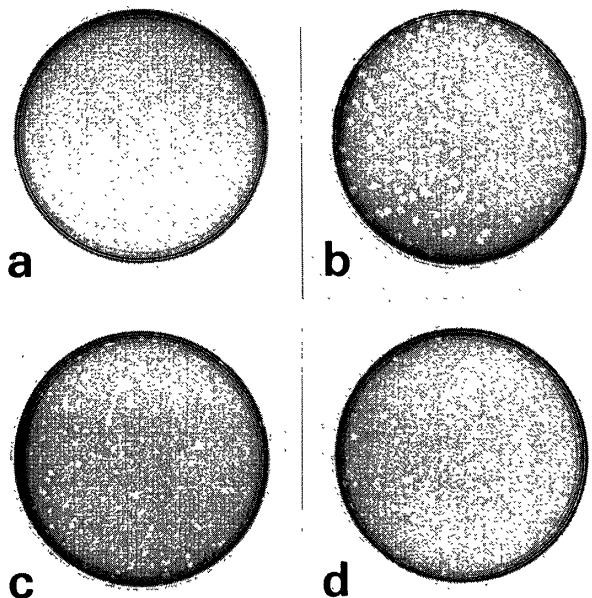


Fig. 1. Appearance of plaques formed by CCV strains in CRFK cell cultures. (a) Mock infected control culture, (b) 1-71 strain, (c) Toda strain, (d) 5821 strain.

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要 約

CRFK 細胞によるイヌコロナウイルスのプラック定量法：土屋耕太郎・笠岡達彦・畔高政行・高橋英司・小西信一郎（東京大学農学部家畜微生物学教室）——CRFK 細胞を用いることにより，3 株のイヌコロナウイルス（1-71株，戸田株，5821株）はウイルス接種後 2 日で明瞭なプラックを形成し，接種ウイルス量とプラック数の間には正の相関関係が成立し，再現性が高く，簡便な定量法が確立された。本法が中和試験に応用できることも確められた。