Plaque Assay for Titration of Bovine Enteric Coronavirus

SUMMARY

The plaquing ability of two isolates of bovine enteric coronavirus (BECV) was studied in HRT18 (human rectal adenocarcinoma) cell monolayers. Both isolates were able to induce plaque formation within 2 to 3 days; plaques appeared as round opalescent areas which remained colourless after neutral red or crystal violet staining. A good correlation was found between the titres as determined either by counting the plaques that were visible to the naked eye before and after neutral red staining, or by enumerating fluorescence or haemadsorption foci.

Bovine enteric coronaviruses (BECV) have been identified as aetiological agents of neonatal diarrhoea in calves (Stair *et al.*, 1972; Mebus *et al.*, 1973; Doughri & Storz, 1977; Bridger *et al.*, 1978*a*; Gouet *et al.*, 1978). Although different isolates have already been adapted either to organ culture (Bridger *et al.*, 1978*b*) or to cell culture (Mebus *et al.*, 1973; Bridger *et al.*, 1978*a*; Dea *et al.*, 1980) very little is known about strain differences that may well exist. This gap in our knowledge is related to two major problems: difficulties in primary isolation of the virus, and lack of practical and reliable procedures to titrate and plaque-purify virus populations. No adequate plaque assay for BECV has been described, although a cytopathic effect was observed in some continuous cell lines (Dea *et al.*, 1980; Inaba *et al.*, 1976). Since HRT18 (human rectal adenocarcinoma) cells have been shown to be highly susceptible to BECV, allowing good multiplication and improved yield of virus (Laporte *et al.*, 1980), we used these cells to study the ability of two French isolates to induce plaque formation. The current report describes the optimal conditions for this assay.

HRT18 cells (Tompkins *et al.*, 1974) were grown in RPMI 1640 medium supplemented with 20% foetal bovine serum (FBS), 100 units/ml penicillin, and 0·1 mg/ml streptomycin (the growth medium). Two isolates of BECV (F15 and G110) were used throughout this study. Both were obtained from the faeces of calves suffering from acute diarrhoea. Isolate F15 was kindly supplied by Dr J. Laporte. This virus was initially adapted to HRT18 cells and subsequently passaged 13 times in the same cell line. Isolate G110 (Laporte *et al.*, 1979), kindly provided by R. L'Haridon, was adapted in primary foetal bovine kidney cells (PBFK) and then serially passaged 34 times in these cells.

Virus stocks of both isolates were prepared by infecting 7-day-old monolayers of HRT18 cells in 75 cm^2 plastic tissue culture flasks (Corning Glass Works, N.Y., U.S.A.). After an incubation period of 3 days at 37 °C, cell cultures were frozen and thawed, and 2 ml amounts of cell fluid suspension stored at −80 °C until use.

All plaque assays were performed in 6-well Costar plates (Costar, Cambridge, Mass., U.S.A.). Confluent monolayers of HRT18 cells were trypsinized with a solution containing 0·6 g/l trypsin (Difco) and 1·2 g/l EDTA. Samples of 1×10^6 freshly trypsinized cells in 2 ml growth medium were added to each well and the plates were incubated at 37 °C for 4 days in a humidified atmosphere containing 5% CO_2. After two washes with RPMI 1640 medium supplemented with antibiotics only, confluent monolayers were inoculated with 0·2 ml of virus serially diluted in RPMI 1640 medium supplemented with 2% FBS and antibiotics. The inoculum was allowed to adsorb for 1 h at 37 °C with manual rocking every 10 min. Monolayers were then washed with RPMI 1640 medium and an agarose overlay was applied

at 39 °C. The agarose overlay consisted of RPMI 1640 medium containing 0·6% agarose (Industrie Biologique Francaise, Clichy, France), 2% FBS, 100 units/ml penicillin and 0·1 mg/ml streptomycin. Plates were then incubated at 37 °C in a CO_2 incubator for 3 days. Mock infection was performed as described above with virus inoculum replaced by RPMI 1640 medium containing antibiotics and FBS (2%). Plaques were revealed by four methods.

(i) Neutral red staining: 2 ml of a 0·01% solution of neutral red in tris–Eagle's minimum essential medium were added to each well and the plates incubated at 37 °C for 3 h in the dark.

(ii) Crystal violet staining: after overnight fixation of the monolayers in a 2·5% glutaraldehyde solution in distilled water, the agarose overlay was discarded and 1 ml of a 1% crystal violet solution in distilled water was added to each well. Plates were kept at room temperature for 3 h and then washed four times with distilled water.

(iii) Haemadsorption: assays were carried out using rat red blood cells (RRBC). Blood collected on Alsiever solution (v/v) was centrifuged at low speed, the supernatant fluid discarded and the RRBC washed twice with Dulbecco's phosphate-buffered saline solution (PBSS). Washed RRBC were diluted to a 2% (v/v) suspension in PBSS supplemented with 2 mg/ml bovine serum albumin (BSA). One ml of the RRBC suspension was added to each well and haemadsorption was allowed to occur at room temperature for 1 h. Before scoring the number of haemadsorption foci, cell monolayers were washed twice with PBSS to remove unadsorbed erythrocytes.

(iv) Indirect immunofluorescence test (IIF): cell monolayers were fixed in ethanol at −20 °C for 30 min; plates were then air-dried and stored at −20 °C before staining. BECV antigens were revealed by an IIF test using first a rabbit anti-BECV serum prepared in our laboratory and second a sheep anti-rabbit Ig serum conjugated to fluorescein isothiocyanate (FITC) (Institut Pasteur Production, Paris, France). The anti-BECV serum was raised in specific pathogen-free (SPF) rabbits by injecting purified BECV (G110 isolate) emulsified in incomplete Freund's adjuvant. In some experiments three calf anti-BECV sera were used instead of the rabbit serum. These sera, kindly supplied by Dr P. W. De Leeuw (Centraal Diergeneeskundig Instituut, Lelystad, The Netherlands) were obtained by infecting three gnotobiotic calves with the Dutch (Van Balken *et al.*, 1978), Danish and British (Bridger *et al.*, 1978a) isolates of BECV. The first step of the reaction was performed as described above and for the second one a FITC anti-bovine IgG was used (Miles-Yeda, Rehovot, Israël).

When HRT18 cells were infected with F15 virus, plaques became visible at 48 h post-inoculation. By this time they appeared as round opalescent areas when observed by indirect illumination against a dark background, and as foci of clustered cells when examined under an inverted microscope. Seventy-two h after inoculation plaques had enlarged but no sign of lysis appeared in the clustered cells. The number of plaques reached a maximum by 2 days and incubation for a further 48 h did not allow any more plaques to appear (Table 1). After neutral red (Fig. 1a) or crystal violet staining, BECV F15 plaques appeared as clear unstained round areas the average diameter of which was 0·6 mm by day 2 post-infection and reached 2 mm after 3 days. No plaques could be seen in control monolayers. It should be mentioned that F15 virus induced plaques after only three serial passages on HRT18 cells.

Since other BECV strains were reported to agglutinate rat, hamster and mouse erythrocytes (Sharpee *et al.*, 1976), we tried to reveal the foci of infected cells by a haemadsorption test using RRBC. Visible plaques were outlined by the adsorption of RRBC on the infected cells (Fig. 1b). This adsorption was considered as specific as no haemadsorption foci could be detected in mock-infected monolayers.
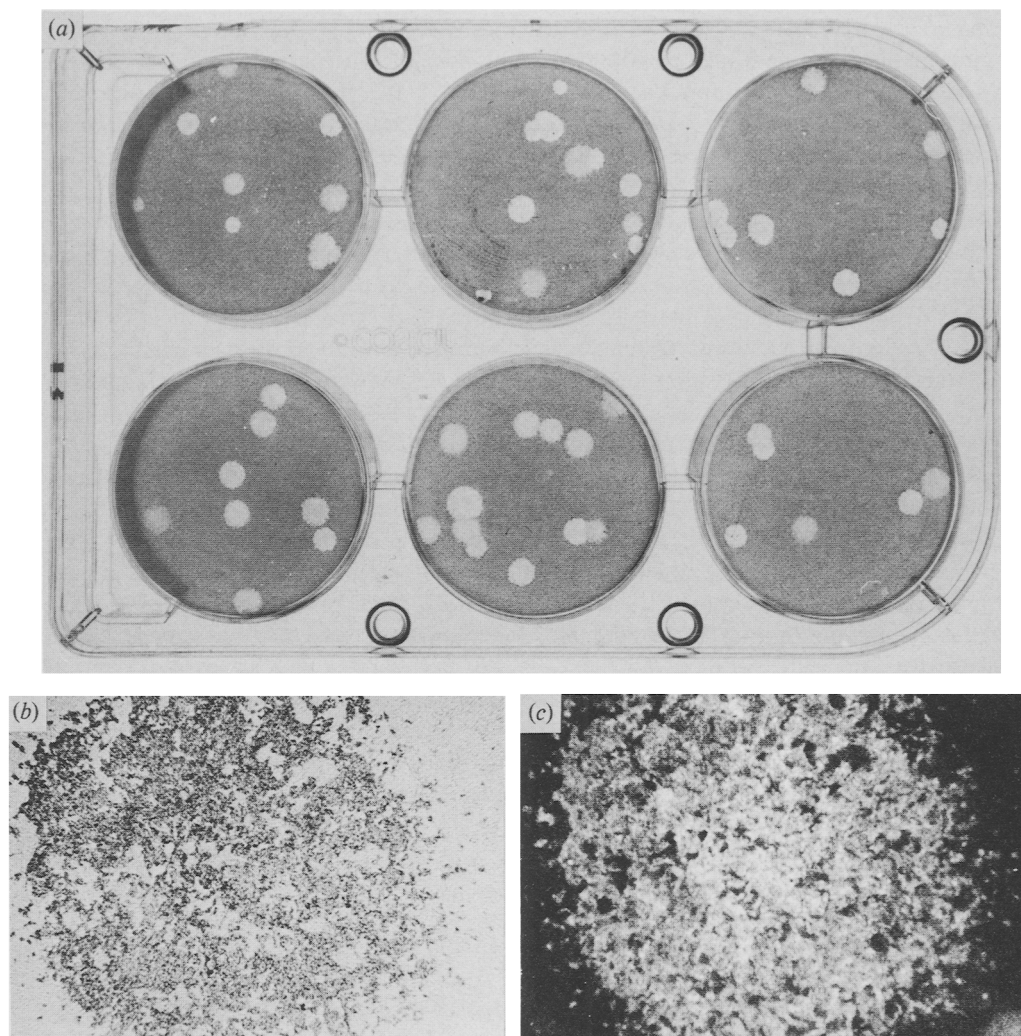
Fig. 1. (*a*) F15 plaques at 72 h post-inoculation and stained with neutral red. Virus dilution is 10^{-6}. (*b*) RRBC adsorbed on an F15 plaque at 60 h post-infection. (*c*) RRBC adsorbed on an F15 plaque at 60 h post-infection and visualized by IIF staining. The monolayers which had been previously allowed to react with RRBC were fixed with ethanol and stained by IIF as in Methods.

Table 1. *Titration of F15 isolates (16th passage): plaque enumeration as a function of dilution factor and time*

		Time		
Assay no.	Virus dilution	48 h	72 h	96 h
	10^{-5}	21·1*	21·1	22
I	10^{-6}	2·8	2·6	2·8
	10^{-7}	0	0	0
	10^{-5}	22·2	24·8	22·6
II	10^{-6}	1·8	2	2·3
	10^{-7}	0	0	0

* Mean no. of plaques per plate (6 wells per dilution), staining omitted.

Finally, the IIF test provided a practical means to identify the BECV F15 isolate as the inducer of visible and haemadsorption plaques. All the visible plaques and haemadsorption foci corresponded to fluorescent areas revealed by IIF (Fig. 1 c). No fluorescence was detected in control monolayers stained by the same technique. The same result was observed with either our rabbit anti-BECV serum or with the anti-BECV sera raised against the Danish, British and Dutch BECV isolates.

The ability of the G110 isolate to plaque in HRT18 cells was first tested using the 34th passage of this virus in PBFK cells. Visible plaques or haemadsorption foci were not induced by this first passage in HRT18 cells, although small areas consisting of 5 to 20 fluorescent cells could be detected at 72 h post-infection. After two serial passages on HRT18 cells, the same virus induced visible plaques which appeared on day 3 after inoculation. These plaques were also detectable after neutral red or crystal violet staining, appearing as faintly stained round areas, measuring 2 mm diam., 72 h post-infection. Haemadsorption and IIF tests confirmed that the visible plaques were induced by BECV. After seven serial passages of BECV G110 on HRT18 cells, no real difference could be observed between plaques produced by F15 and G110 isolates.

As shown in the present work, HRT18 cells constitute a suitable system for BECV titration by plaque assay. This assay is simple, as it does not require complex overlay media or multiple overlays. Furthermore, once adapted to HRT18 cells, plaques become clearly visible without any staining on day 2 to 3 post-infection allowing a rapid and reliable determination of virus titres. In our experience HRT18 cells provided good confluent monolayers without discontinuities which could be confused with plaques. The plaque assay described here meets the criteria for a useful test (Cooper, 1967), being sensitive, reproducible and quantitative. This test enables plaque purification and genetic studies to be conducted and provides a practical tool for sero-epidemiological work on the bovine enteric coronaviruses.

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