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Short communication

A carboxymethyl-cellulose plaque assay for feline calicivirus

Jaime Escobar-Herrera, Fernando José Medina-Ramírez, Ana Lorena Gutiérrez-Escolano *

Department of Experimental Pathology, Centro de Investigación y de Estudios Avanzados del IPN (CINVESTAV-IPN), Av. IPN 2508, Col. San Pedro Zacatenco, C.P. 07360 México, D.F. Mexico

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Abstract

The standardization of a plaque assay for feline calicivirus in Crandell Reese feline kidney cells using carboxymethyl-cellulose as an overlay medium is described in this report. This methodology gives comparable counts as compared to the standard assay, and prevents monolayer roll over and peel off, as well as easy medium removal. Cell fixation and staining is performed in a considerably reduced period of time, compared to agarose-based methods.

Keywords: Feline calicivirus; Plaque assay; Carboxymethyl-cellulose; Crandell Reese feline kidney cells; Gastroenteritis

The family *Caliciviridae* comprises a variety of different human and animal pathogens. The human caliciviruses belong to the genera *Norovirus* and *Sapovirus*, whereas the genera *Lagovirus* and *Vesivirus* contain only animal pathogens. Human caliciviruses are recognized worldwide as the most common cause of foodborne and waterborne outbreaks of acute gastroenteritis in children and adults (Frankhauser et al., 1998; Green et al., 2001). Recently, these enteric viruses have also been associated with sporadic cases of gastroenteritis (Widdowson et al., 2005). Incidents of the rapid spread of human caliciviruses-related illness in hospitals, nursing homes, military settings, restaurants, and cruise ships have lead to an increased recognition of the impact of these viruses on public health.

The study of the biology of human caliciviruses has been hindered by the lack of cell culture systems. However, feline calicivirus, a member of the genus *Vesivirus*, which is easily grown in tissue culture and shares many of the biological properties of human caliciviruses, has been used as a model for studying some of the general replication strategies.

The feline calicivirus can grow readily and produces clear cytopathic effects in culture cell lines, particularly in Crandell Reese feline kidney cells; therefore, its propagation and quantification by tissue culture infectious dose ($TCID_{50}$) and plaque assay have been extensively reported. Different procedures and reagents, such as agar (Kalunda et al., 1975) or agarose incorporated in the overlay media, have been used for plaque assays (Bidawid et al., 2003). The standardization of the agarose plaque assay for feline calicivirus in Crandell Reese feline kidney cells has brought a sensitive and reproducible method for virus titration; this method has also been adapted to other cultivable caliciviruses such as the murine norovirus in RAW 264.7 cells (Hsu et al., 2005) and porcine enteric calicivirus in LLC-PK cells (Chang et al., 2005). However, the Crandell Reese feline kidney cell monolayer is often fragile and has a tendency to roll over and peel off the flask's surface (Bidawid et al., 2003). Therefore, when the agarose is removed, the monolayer is commonly damaged, resulting in variations on plaque quantification. An interesting alternative is the use of carboxymethyl-cellulose, which increases the viscosity of the overlay medium preventing virus spreading; furthermore, it is easily removed from the tissue culture plates. Carboxymethyl-cellulose as an overlay medium has been broadly used in plaque assays of different viruses in several cell lines (Triantafilou and Triantafilou, 2004; Cologna and Rico-Hessle, 2003; Bae et al., 2003; Oien et al., 2002). In this article, we report the standardization of a feline calicivirus titration method in Crandell Reese feline kidney cells using carboxymethyl-cellulose. The use of carboxymethyl-cellulose in our system brings the advantage of smooth removal of the overlay medium, preventing monolayer damage. Additionally, cell fixation and staining of the monolayer after carboxymethyl-cellulose removal is performed in a considerably reduced period of time, compared to the agarose method.

* Corresponding author. Tel.: +52 5061 3800x5677; fax: +52 5061 3377.
E-mail address: alonso@cinvestav.mx (A.L. Gutiérrez-Escolano).

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Plaque assays were undertaken using monolayers of Crandell Reese feline kidney cells obtained from the American Type Culture Collection (Rockville, MD), propagated in minimum essential medium containing 10% horse serum (HS) and supplemented with antibiotics, at 37 °C in humidified 5% CO_2 incubator. 5×10^4 cells were seeded in 12-well tissue culture plates and allowed to adhere for 24 h, after which, cells were washed with minimum essential medium without serum, and tenfold serial dilutions of feline calicivirus (F9 strain, obtained from the American Type Culture Collection) (Rockville, MD) in minimum essential medium without serum, were inoculated in duplicate or triplicate and incubated for 1 h at 37 °C. The overlay medium using carboxymethyl-cellulose was prepared during viral adsorption as follows: 2× carboxymethyl-cellulose (Sigma) solutions were prepared at concentrations of 1.6%, 2.0% and 2.4% (w/v) in water, and stirred for 1 h at room temperature (RT). After this time, equal volumes of 2× supplemented—minimum essential medium with 10% HS and antibiotics and each of the 2× carboxymethyl-cellulose solutions were mixed separately and incubated at 37 °C until needed. At the end of the virus incubation period, the viral inoculum was aspirated, the cells were washed once with minimum essential medium and 400 μl of each carboxymethyl-cellulose mixture was added to each well and incubated for 30 h at 37 °C in a humidified 5% CO_2 incubator. Finally, the overlay medium was aspirated; cells were washed with minimum essential medium, and fixed for 15 min with 250 μl of a 3.7% solution of formaldehyde in PBS prepared just before its use. Plaques were visualized by staining for 10 min at RT with 0.1% (w/v) with crystal violet in 20% (v/v) ethanol. For parallel plaque assays using overlay medium, equal volumes of 2× supplemented—minimum essential medium with 10% HS, antibiotics and 2× agarose (Invitrogen) (1.2% (w/v) in water) were prepared during the virus adsorption period and stored at 43 °C until used (Bidawid et al., 2003). After the cells were washed, the same volume of both solutions described above were mixed and 400 μl of the mixture was added to the monolayer; it was allowed to solidify at RT and incubated at 37 °C for 30 h. After this time, monolayers were fixed overnight (shorter fixation times resulted in monolayer peel off) by adding 250 μl of 3.7% solution of formaldehyde in PBS per well. Subsequently, formaldehyde was discarded and the agarose was removed under a gentle steam of cold running water. The plates were left to dry at RT and the cells were stained with crystal violet solution as described above.

To determine if the carboxymethyl-cellulose, as an overlay medium, allows the formation of plaques with feline calicivirus, monolayers of Crandell Reese feline kidney cells were infected with 125.5, 24.9 and 12.45 pfu/well of feline calicivirus (Fig. 1I–III, respectively). Small homogeneous plaques were observed with all three of the different concentrations of carboxymethyl-cellulose (0.8%, 1.0% and 1.2%) used; however, the plaque size at 1.2% carboxymethyl-cellulose was even smaller than the plaques observed at 0.8% and 1.0% of carboxymethyl-cellulose (Fig. 1). In general, plaque size was smaller with carboxymethyl-cellulose than with agarose. It is important to note that in the presence of carboxymethyl-cellulose, monolayer integrity was well conserved even though

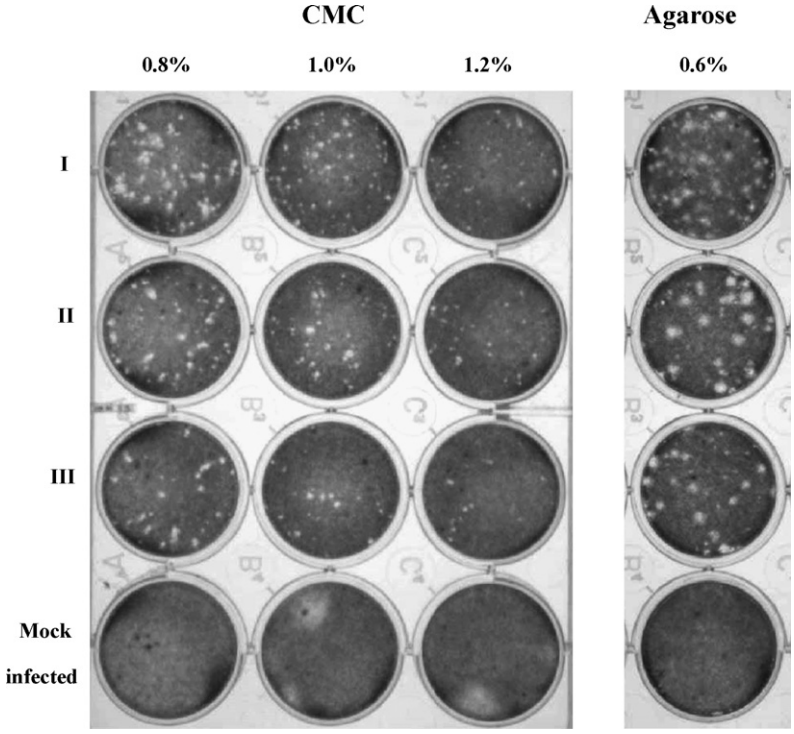

Fig. 1. Parallel plaque assays under 0.6% agarose or 0.8%, 1.0% and 1.2% of carboxymethyl-cellulose overlays. Crandell Reese feline kidney cells were mock infected or infected with 125.5, 24.9 and 12.45 pfu/well of feline calicivirus (left column, I–III, respectively). After 1 h incubation, carboxymethyl-cellulose at 0.8%, 1.0% and 1.2% or agarose at 0.6% was used as an overlay medium for 30 h at 37 °C. Cultures were fixed and stained with crystal violet.