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Short communication

Assessment of plaque assay methods for alphaviruses

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A B S T R A C T

Viruses from the *Alphavirus* genus are responsible for numerous arboviral diseases impacting human health throughout the world. Confirmation of acute alphavirus infection is based on viral isolation, identification of viral RNA, or a fourfold or greater increase in antibody titers between acute and convalescent samples. In convalescence, the specificity of antibodies to an alphavirus may be confirmed by plaque reduction neutralization test. To identify the best method for alphavirus and neutralizing antibody recognition, the standard solid method using a cell monolayer overlay with 0.4% agarose and the semisolid method using a cell suspension overlay with 0.6% carboxymethyl cellulose (CMC) overlay were evaluated. Mayaro virus, Una virus, Venezuelan equine encephalitis virus (VEEV), and Western equine encephalitis virus (WEEV) were selected to be tested by both methods. The results indicate that the solid method showed consistently greater sensitivity than the semisolid method. Also, a "semisolid-variant method" using a 0.6% CMC overlay on a cell monolayer was assayed for virus titration. This method provided the same sensitivity as the solid method for VEEV and also had greater sensitivity for WEEV titration. Modifications in plaque assay conditions affect significantly results and therefore evaluation of the performance of each new assay is needed.

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Alphaviruses circulate widely in the world and cause disease, often affecting either the musculoskeletal or the central nervous system. In South America, the main alphaviruses that circulate are Venezuelan equine encephalitis virus (VEEV), Eastern equine encephalitis virus (EEEV), Western equine encephalitis virus (WEEV), Mayaro virus (MAYV), Una virus (UNAV) and Trocara virus (TROCV) (Aguilar et al., 2004; Forshey et al., 2010; Powers et al., 2006; Travassos da Rosa et al., 2001; Turell et al., 2005). While VEEV subtype IC and IAB are mainly associated with major epidemics that reach high rates of mortality in equines, enzootic subtype ID has been isolated repeatedly from humans and mosquitoes recently (Aguilar et al., 2009). MAYV produces dengue-like acute febrile illness including arthralgia and arthritis. Serosurveys suggest that MAYV is endemic across the Amazon Basin, and isolates have been obtained from humans, monkeys and mosquitoes (Forshey et al., 2010; Lavergne et al., 2006; Powers et al., 2006; Tesh et al., 1999). Despite the lack of apparent human cases of EEEV, WEEV, UNAV and TROCV in South America, these viruses have been isolated repeatedly from mosquitoes, birds and other vertebrates, remaining in an often poorly understood, natural-cycle (Aguilar et al., 2007; Powers et al., 2006; Travassos da Rosa et al., 2001).

Several studies have described arbovirus distribution, vector transmission, host–pathogen interaction, and public health impact of arboviruses (Forshey et al., 2010; Gould et al., 2010; Morrison et al., 2008; Turell et al., 2005; Weaver and Reisen, 2010). Studies such as these often use plaque assays or plaque reduction neutralization tests (PRNTs) to measure the amount of virus or antibodies present in a sample. Both plaque assays and PRNTs are considered gold standards in virus and antibody detection, respectively; however, these tests should be standardized and evaluated for performance and reproducibility for specific viruses, especially when data are shared between laboratories.

Since the first description of plaque assays (Dulbecco, 1952), modifications to the original approach have been made, including the use of different substances in the overlay media and different cell preparations (Hotchin, 1955; Matrosovich et al., 2006; Morens et al., 1985; Schulze and Schlesinger, 1963). Agar or agarose have been used widely in standard plaque assays as a solid overlay (Aguilar et al., 2004; Beaty et al., 1989; Henderson and Taylor, 1959). However, an alternative semisolid method using carboxymethyl cellulose (CMC) has been reported to possess advantages over the traditional agar overlay including: (1) easy medium removal and (2) prevention of monolayer peel off. Consequently, this approach has been adopted for many viral assays (Morens et al., 1985; Rapp, 1963; Schulze and Schlesinger, 1963).

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Table 1
Viruses used in the study.

Virus strain	Passage history
MAYV – TRVL 15537	2 passages in Vero cells
UNAV – PE10800 TVP5402	3 passages in Vero cells
VEEV – TC83	83 passages in Guinea pig heart cell/and three passages in Vero cells
WEEV – VR70	2 passages in Vero cells

Any modification of the plaque assay and its possible impact on virus titration or neutralizing antibody titers should be established carefully. Modifications in assay conditions have been shown to impact flavivirus results significantly (Thomas et al., 2009); however, the extent plaque assay conditions affect alphavirus diagnosis is unknown. In an attempt to provide information that could aid researchers in selecting appropriate alphavirus plaque assay and PRNT conditions, a series of experiments using VEEV, MAYV, UNAV and WEEV were conducted to evaluate the variability of viral titer and titer of neutralizing antibody under two standard methods: a solid method using cell monolayer under agarose overlay and a semisolid method using a cell suspension under CMC overlay. Finally, a semisolid-variant method was evaluated for examining the effects of cell preparation from the type of overlay medium used.

Both Vero-76 and BHK-21 cells are used commonly for alphavirus plaque assay and plaque reduction neutralization tests. In this study, Vero-76 cells were selected because plaque morphology was defined clearly compared to those in BHK-21 cells allowing a reliable counting of plaques (data not shown). Vero-76 cells were propagated at 37 °C in Eagle's minimum essential media (EMEM) supplemented with 10% of heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin. The study was performed using the alphavirus strains listed in Table 1. All cell lines had an initial passage number of 40.

A description of the main steps in the plaque assay methods are shown in Fig. 1. For the solid method, cell suspension was seeded with 3×10^5 cells in a 12-well plate and incubated at 37 °C in 5% CO_2. Upon reaching ~80% confluence, growth media was removed and 100 μl of 10-fold virus dilutions from 10^1 to 10^9 were added and incubated for 1 h, with rocking every 15 min to allow virus adsorption. A 3 ml overlay consisting of 0.4% agarose in EMEM (supplemented with 2% of heat-inactivated FBS and 1% penicillin/streptomycin) was added, and the plates were incubated at 37 °C for 48 (UNAV, VEEV and WEEV) or 72 h (MAYV). After incubation, agar plugs were removed, and the cells were fixed and stained with 0.1% napthol blue black, 1.6% sodium acetate in 6% glacial acid acetic for 30 min. For the semisolid method, 3×10^5 cells were seeded to 12-well tissue culture plates and 100 μl of 10-fold virus dilutions from 10^1 to 10^9 were added immediately to the cells, with no rocking. Virus inoculation was performed simultaneously from the same virus dilution under each of the conditions. Cells were incubated for 3 h to allow virus adsorption and, after incubation, a CMC overlay media consisting of 0.6% carboxymethylcellulose, EMEM without phenol red, 10% FBS, 0.075% $NaHCO_3$ and penicillin/streptomycin was added. Plates were incubated at 37 °C for 48 (UNAV, VEEV and WEEV) or 72 h (MAYV). After incubation, cell overlays were removed, and the cells were fixed and stained as describe above. For the semisolid-variant method, plates containing 80% confluent Vero monolayers were used as described in the solid method, but with the addition of a CMC overlay media. After incubation, cell overlays were removed, and the cells were fixed and stained as described above.

For use as positive controls in PRNT, hyperimmune mouse ascitic fluid (HMAF) was obtained by immunizing adult mice with virus mixed with Freud's adjuvant and then injecting them with sarcoma cells. Two sets of human serum were analyzed to determine antibody neutralization titers under solid and semisolid conditions. First, nineteen paired acute and convalescent serum samples were obtained through a febrile surveillance study. All paired samples were from patients that had MAYV isolated from their acute sera. All convalescent samples were collected between one week and three months after the onset of symptoms, as described previously (Forshey et al., 2010). Second, serum samples were

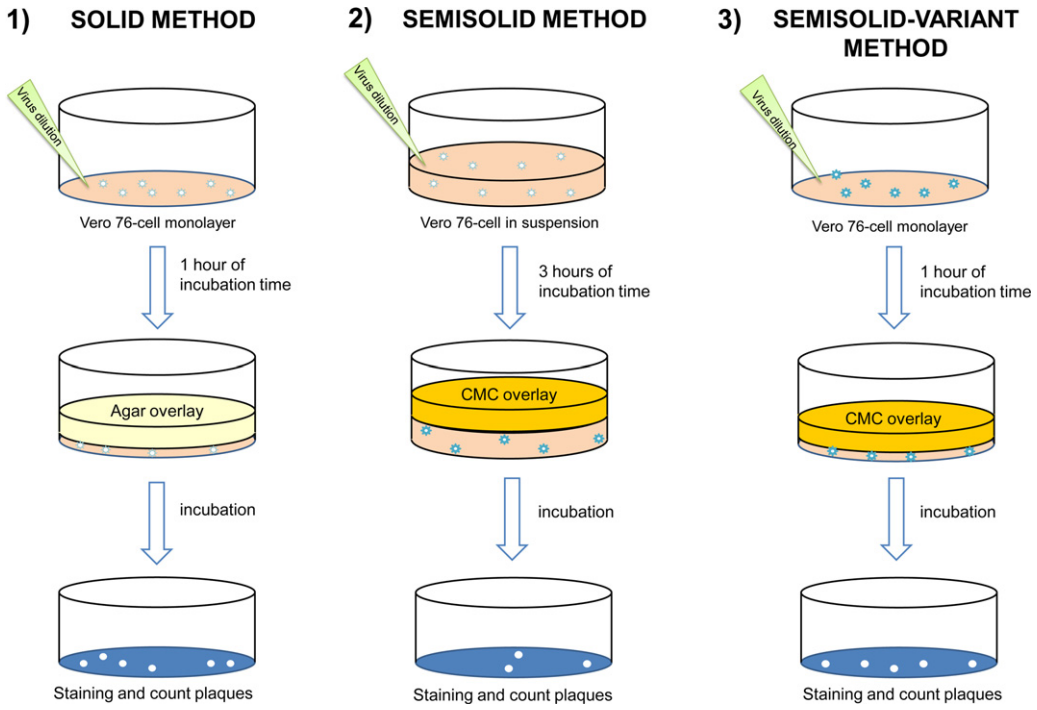

Fig. 1. Three methods used for alphavirus and antibody titration.

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