

Research Article

Performance of methylcellulose and Avicel overlays in plaque and focus assays of Chikungunya virus

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Background: Chikungunya virus is a re-emerging pathogen that is responsible for Chikungunya fever periodic outbreaks along the Kenyan coast and in other African countries. Epidemiological data from the World Health Organization show that in 2014-2015, there was a major outbreak of Chikungunya fever in the Americas and Pacific Islands. Surveillance and correct diagnosis are therefore key in controlling the spread and management of the disease.

Plaque and focus assays are key techniques in viral characterization or quantification, and both assays typically require overlay with gelling polymers to limit the spread of viruses in cell culture. There are anecdotal reports that Avicel may be superior to methylcellulose in assay of Influenza virus. However, it is unclear whether this would apply to other viruses.

Objective: The objective of this study was to determine the performance of methylcellulose and Avicel overlays in plaque and focus assays of Chikungunya virus.

Methods: Confluent Vero cells were seeded in 6- or 96-well plates for plaque and focus assays respectively. Cells were inoculated with serially diluted Chikungunya virus, and incubated to allow adherence of the virus to the cells. The inoculum was removed; replaced with Avicel or methylcellulose overlay at various concentrations and stained with crystal violet or immunostained. Statistical significance was computed using the Holm-Sidak test.

Results: The size of plaques formed by Chikungunya virus was dependent on the concentration of both Avicel and methylcellulose gels used as overlays, with Avicel overlays giving consistently larger plaques than methylcellulose. Chikungunya virus formed plaques nearly 2.5 times larger in diameter (2 vs 0.8 mm) with 1.2 % Avicel than with 1.25 % methylcellulose after 60 hr growth. Plaques formed with Avicel were better defined and easier to count after 48 hr growth period compared to a 60 hr period. However, methylcellulose overlays provided smaller, more distinct and better defined foci in focus assays.

Conclusion: Both methylcellulose and Avicel are good overlay media for viral assays. Avicel is marginally better for plaque assays while methylcellulose provides more distinct and easier to count foci in focus assays.

Key words: Chikungunya virus, plaque assay, focus assay, methylcellulose, Avicel

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1. Introduction

Chikungunya virus (CHIKV) is an arthropod-borne alphavirus that belongs to the *Togaviridae* family, and is responsible for sporadic worldwide outbreaks of Chikungunya fever (CHIKF) (Charrel et al, 2007; Nasci, 2014).

According to the World Health Organization (WHO), there was in 2014/2015 a CHIKF outbreak in the Americas and Pacific Islands, affecting more than 40 countries, with more than 1 million suspected cases (Leparc-Goffart et al, 2014; Khan et al, 2014; WHO, 2015).

CHIKV is primarily transmitted to humans through the bite of infected female *Aedes* mosquitoes (Jupp and McIntosh, 1988). Early Chikungunya fever symptoms include high fever, myalgia, headache and rashes. These are followed in the late phase by intense arthralgia, severe headaches, seizures, sensory abnormalities and motor dysfunction that gives a characteristic contorted posture (or *chikungunya* in the Kimakonde language), from which the disease derives its name (Tournebize et al, 2009; WHO, 2015).

Chikungunya fever is an important emerging infection in tropical Africa, south-east Asia, central and south America, with no antiviral medicines nor vaccines yet available. Diagnosis and surveillance of CHIKV are essential to prevent its spread, but are challenged by insufficient availability of cost effective diagnostic tools and treatment options (Chipwaza et al, 2014; WHO, 2015). High through-put methods for viral quantification are therefore essential in supporting development of novel treatments, vaccines and diagnostic tools.

CHIKV infectious particles can be quantified using plaque assays to measure its cytopathic effect (CPE) in high through-put 96-well microtitre plates. Plaque assays are the standard method for quantifying viral infectious particles (Pickard et al, 2009). A viral plaque is a clear area of lysed cells formed by viruses growing on a monolayer of confluent host cells. Plaques are useful for the determination of viral titre of a liquid sample, as each plaque represents an infectious virus particle. For viruses that do not lyse cells, or to avoid the long infection times required for plaque assays, focus assays can be performed. In focus assays, viral antigens expressed by host cells are immunostained with labelled antibodies, and foci (rather than plaques) are visualized and counted (Flint et al, 2009).

During plaque assays, a confluent monolayer of host cells is infected with serially diluted viral samples, and the host cells are covered with an overlay medium to limit the spread of the viral particles. The overlay medium is typically made of semi-solid or gel forming polymers such as agarose, agar, methylcellulose or Avicel with sufficient viscosity to optimally reduce viral diffusion. The choice of overlay medium is thus critical for the success of the assay, and in this regard, Matrosovich et al (2006) demonstrated that Avicel was superior to methylcellulose and agar overlays for Influenza viral plaque and focus assay. In this study, we compared the performance of methylcellulose, a commonly used overlay medium, and Avicel as overlay media in CHIKV plaque and focus assays.

2. Materials and Methods

2.1 Viruses and Cells

Vero cells (American Type Culture Collection - ATCC, CCL81) were cultivated in single strength Eagle's Minimum Essential Medium (EMEM) (Gibco, Paisley, UK) supplemented with 2 % L-Glutamine (Sigma-Aldrich, St. Louis, USA), 10 % Fetal Bovine Serum (Sigma, St. Louis, USA), 2 % Penicillin-Sterptomycin (Penicillin 1000 U/mL, Streptomycin 10mg/mL (Sigma-Aldrich, St. Louis, USA) and 1 % non-essential amino acids (Gibco, Paisley, UK). Cells were incubated at 37°C till a 90 %-confluent monolayer was observed.

Chikungunya virus (CHIKV strain Lamu 33) is a well characterized strain isolated from a patient from Lamu, Kenya during the 2004 Chikungunya outbreak (Kariuki Njenga et al, 2008). To obtain viral stock, confluent monolayers of Vero cells in 75 cm² culture flasks were infected with CHIKV, incubated at 37 °C under a 5% CO₂ atmosphere and harvested on Day three when about 90 % of CPE was attained. The infected culture fluid (ICF) was harvested by centrifuging at 600 *g* for 10 min in a refrigerated centrifuge (TOMY Model AX-311, Tokyo, Japan), and the supernatant (the viral stock) aliquoted and stored at - 80°C.

2.2 Infection of cells for plaque and focus assays

Tenfold serial dilutions (1x10¹ to 1x10⁷) of the virus stock were prepared in EMEM containing 2 % Fetal Calf Serum and inoculated into their respective wells in 6-well or 96-well plates seeded with 90% confluent monolayer of Vero cells. The plates were incubated at 37 °C in a 5% CO₂ incubator for 90 min to allow adherence of the virus to the cells. The inoculum was removed by aspiration and replaced with 3 ml of Avicel or methylcellulose overlay at various concentrations. The plates were incubated for 60 and 28 hr for plaque and focus assays respectively. Plaques were stained with 1 % crystal violet stain after fixing with 3.7 % formaldehyde for 2 hr at room temperature. The foci were immunostained with 3,3'-Diaminobenzidine (DAB). Wells were photographed by Nikon camera and the images were acquired in Adobe Photoshop software (Adobe Systems Inc., San Jose, CA, USA).

2.3 Statistical Analysis.

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, USA). Comparisons of radial dimensions of viral plaques and foci were done using the Holm-Sidak repeated-measures analysis of variance (Holm, 1979). A *p* value of < 0.05 was considered significant.

2.4 Ethical considerations

This study was approved by the Kenyatta National Hospital and University of Nairobi Ethics and Research Review Committee (Ref No: **KNH-ERC/A/132**).

3. Results

Plaques are formed by localized damage of host cells due to growth of the virus from one progenitor virus. The destroyed cells do not take up the crystal violet stain, and remain as clear zones upon staining. The size

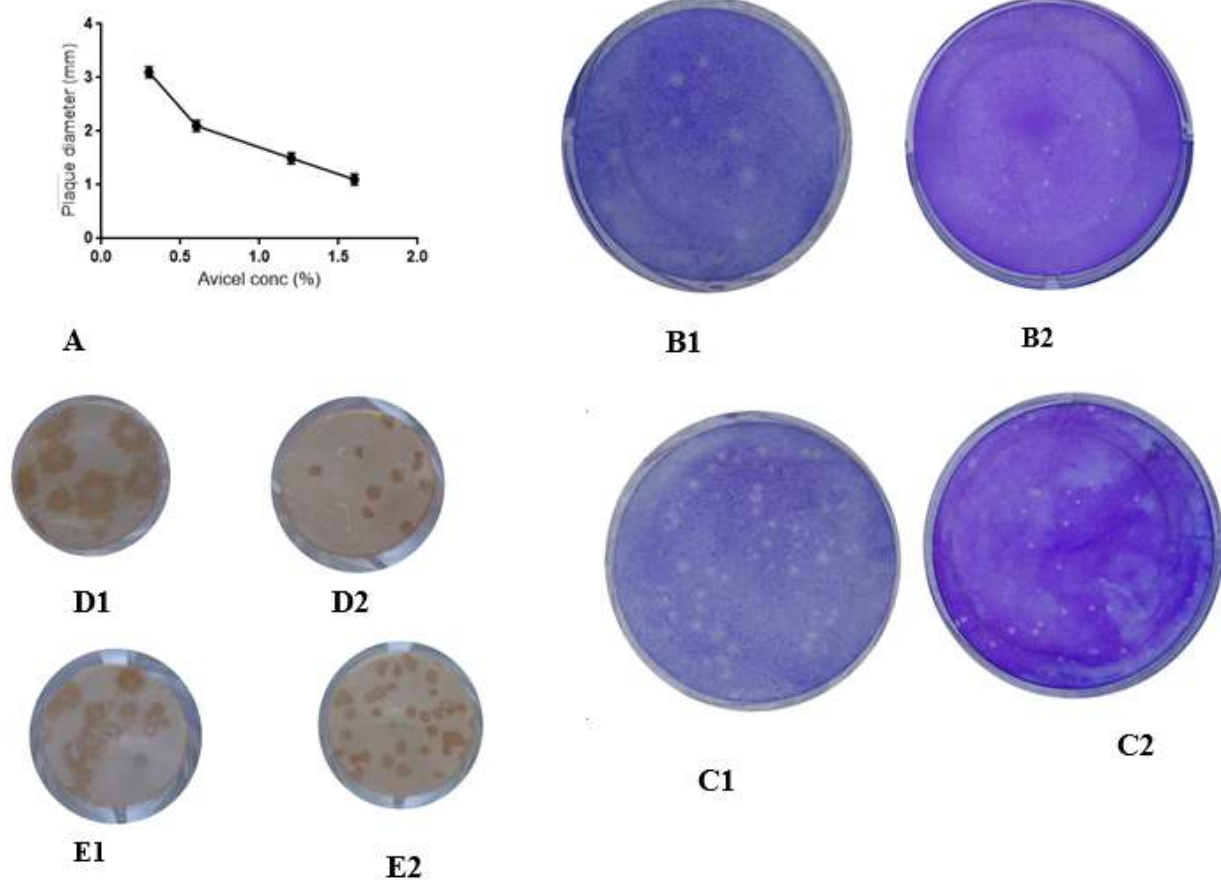
of the plaques is dependent upon the rate of spread of growing virus to the surrounding cells, which is in turn limited by the viscosity of the overlay medium. In the absence of any overlay, the progeny viruses migrate to the surrounding host cells; there is consequently no localized damage, and no plaques are formed.

Our results show that the size of plaques formed by CHIKV were inversely proportional to the concentration of both Avicel and methylcellulose gels used as overlays. Under 0.6 % Avicel overlay CHIKV formed plaques nearly 3 times larger in diameter (3 vs 1.2 mm; $p = 0.0001$) than under 1.6 % Avicel after 48hr growth (**Figure 1**).

Viral plaques formed under methylcellulose were considerably smaller in diameter than those under Avicel across all the concentrations tested (0.3, 0.6, 1.2 and 1.6% for Avicel versus 1.2 and 1.6% for methylcellulose); with the largest plaques obtained

under methylcellulose overlay about two times smaller than the smallest plaque size obtained under Avicel (plaques of 0.71 mm diameter with 1.2% methylcellulose vs 1.2 mm with 1.6 % Avicel; $p = 0.0001$). The plaque sizes under methylcellulose showed little variation at 1.2 and 1.6 % concentration (0.71 vs 0.67 mm diameters; $p = 0.003$). Morphologically, the plaques obtained with both overlays were rounded and clear areas surrounded by the purple region that took up the crystal violet stain (**Figure 1**).

In the focus assays, the foci formed under methylcellulose overlay appeared as dark brown and uniformly stained opaque circular spots against a pale brown translucent background. The edges of foci obtained with methylcellulose overlay were sharper and more distinct than those formed under Avicel, which tended to have speckled focal areas with uneven edges (**Figure 1**).



A. Vero cells were infected with CHIKV Lamu 33 Strain. Cells were stained with 1 % crystal violet dye and diameter of plaque diameters measured for various dilutions of Avicel overlay suspension. The plaque diameter changed inversely to increased concentration of Avicel.

B. Parallel plaque assays at viral titres of 10^{-6} in 6-well plates under Avicel 1.2 % (B1) and methylcellulose 1.2% (B2).

C. Parallel plaque assays at viral titres of 10^{-4} in 6-well plates under Avicel 1.2 % (C1) and methylcellulose 1.2% (C2). Plaques formed under Avicel were generally larger in diameter.

D. Parallel focus assays at viral titres of 10^{-6} in 96-well plates using Avicel 1.2% (D1) and methylcellulose 1.2% (D2). Viruses were immunostained with DAB.

E. Parallel focus assays at viral titres of 10^{-4} in 96-well plates using Avicel 1.2 % (E1) and methylcellulose 1.2% (E2). Viruses were immunostained with DAB.

Figure 1: Performance of methylcellulose and Avicel overlays for plaque and focal assay of Chikungunya virus. Performance was evaluated as plaque diameter and distinctness of focal areas.

4. Discussion

Our results demonstrate that Avicel performed better than methylcellulose overlay in plaque assays as it provided consistently larger plaques which are easier to count. Methylcellulose overlay was, however, more reliable in focus assays, yielding well defined foci, and is therefore more suitable for focal assays in 96 well plates. The differential performance of overlay media has been observed by other researchers as well. For example Herzog et al (2008) showed that Avicel overlay was more suitable for human corona virus plaque assays as it formed larger plaques while agarose overlay was more suitable for purification of the virus. The variations in the performance of different overlay media could be attributed to the nature of the virus and the host cells, as the infectivity of a virus depends on its virulence factors and the receptors on the cell membrane of the host cells. A high the rate of adsorption of a virus to the host cells, leads to the large radial dimension of viral plaque (Koch, 1964; Alvarez et al, 2007). Other factors that may affect the diameter of the plaques and foci include the density and viscosity of the overlay media (Randhawa et al, 1977; Abedon and Yin, 2009). Thus, McKimm-Breschkin (2004) found that carboxymethylcellulose based overlays gave comparatively smaller plaques than agarose when used for plaque assays of respiratory syncytial virus (RSV).

5. Conclusion

Our results demonstrate that Avicel overlay performed better in Chikungunya plaque assays, giving consistently larger plaques that were easier to count. On the other hand, methylcellulose overlay was more suitable for focus assays as it provided sharply defined and distinct focal areas. We therefore recommend Avicel overlay for CHIKV plaque assays and methylcellulose for focus assays.

Conflict of Interest Declaration

The authors declare no conflict of interest.

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