2017-2018 ASM SUMMER SCHOLARSHIP PROJECT:

Tropism of a novel alphavirus

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Abstract

Alphaviruses are the most clinically important endemic viruses in Australia and have a wide range of possible reservoir hosts and vectors. A novel alphavirus was isolated in 2011 from *Culex annulirostris* mosquitos in the Kimberley region of Western Australia. The novel virus, Derby virus (DERV), clusters with the Old-World arthritogenic alphaviruses and is a close relative of Sindbis virus. Important features of DERV, that are yet to be characterised, are its possible reservoir hosts and its potential to cause human infections. This study was conducted to determine the tissue tropism of DERV by using human and avain cell lines as a model of *in vivo* infection. If DERV infects human or avian cell lines it will illustrate the possibility of human or avian infection. Derby virus viral activity was greatest between 1 hour and 24 hours post infection, pointing to a rapid period of viral replication. Derby virus induced plaque formation in two human cell lines; HeLa and HFF and one avian cell line; DF-1 embryonic fibroblasts. Immune cell lines; PMA induced U937 macrophages, SKW and Jurkat cells were not susceptible to Derby virus infection. In combination with preliminary serological evidence these findings indicate that it is possible that Derby virus may infect humans and birds.

Introduction

Mosquito-borne viruses exert a significant burden of disease in Western Australia and are the most clinically important endemic viruses in Australia (1) (2). Outbreaks of Ross River Virus (RRV) and Murry Valley Encephalitis Virus (MVEV) disease following mosquito and reservoir host population changes have illustrated the necessity for surveillance and an understanding of arboviral infection cycles (1,3). Following a particularly wet season in 2010/2011 an outbreak of MVEV involving one fatality occurred (1). During this wet season a viral isolate, K73164, was isolated from *Culex annulirostris* and was initially identified as MVE through monoclonal analysis. This supposed MVE isolate had a markedly different replication time in Vero cells, producing full CPE in 2 days, rather than the expected 7-10 days (4). When the isolate was included in a Next-Generation Sequencing (NGS) run, it was found to be a novel alphavirus; subsequently named Derby virus (DERV) (4).

Alphaviruses are clinically significant and widespread in WA. Ross River virus (RRV) and Barmah Forest virus (BFV) are the most commonly isolated alphaviruses in WA (5) . Many infections are asymptomatic but persistent arthritic and rheumatic symptoms account for significant morbidity in the Western Australian population (6). Australia-wide, up to 7000 infections are attributed to RRV and BFV per annum (5). Although individuals in the northern regions of Australia are at greatest risk of infection, most infections occur in peri-urban regions, across the state (2). RRV and BFV are vectored by a range of mosquito species that exist and breed in a wide range of environments and climates; this contributes to their broad distribution. Climate change will potentially increase mosquito distribution and greater peri-urban developments will increase the likelihood of mosquito-human interactions (7) (8). Subsequently, this raises the risk of human infection (9) (10). This necessitates continued surveillance and understanding of circulating arboviruses, as the knowledge gained will be essential in providing efficient outbreak prevention strategies.

DERV is yet to be fully characterised in terms of its reservoirs hosts, and its potential to cause human infections (4). This study has taken initial steps to characterise DERV by assessing DERV tissue tropism. Evidence of DERV tropism for human or animal cell lines will determine the potential of DERV to infect humans *in vivo* and will lay the ground work for further studies characterising its infection cycle, reservoir hosts and possible human pathogenesis.

Previous emerging viral outbreaks have been characterised through the use of human cell lines as a proxy for human infection (11). This study also utilised cell lines to determine the possibility for human infection from DERV. Cell tropism will be determined by inoculating DERV with various cell lines. Cell lines used in this study were chosen based on their susceptibility to other alphaviruses or likelihood of infection given the pathophysiology of arthritogenic alphaviruses. Jurkat, SKW, U937 induced-macrophages, HeLa, HFF and DF-1 cell lines were incubated with DERV for 24hrs then washed. Aliquots of supernatant were taken every 24-hours for 7 days and subsequently tested for evidence of viral replication using the Haemagglutination assay (HA), Plaque assay (PA) and Focus Forming assays (FFA).

Methods

Jurkat, SKW, U937, HFF, DF-1 and HeLa cells were removed from liquid nitrogen storage (-180°C) and cultured until they replicated reliably (Table 1). Culture protocols were obtained from the ATCC and Imrie Group laboratory's standard operating procedures. For infectivity assays, 100,000 cells were added to 24 well plates in duplicates with controls. Infection wells were inoculated with DERV strain K73164 for 24hrs. After the viral inoculum was removed, the cells were washed three times (with blank media) and aliquots were taken at 24-hour intervals for 7 days, equal volumes of media were added back to the plate. Photographs were taken every 24 hours to monitor for cytopathic effect (CPE), and were compared to the virus free control wells. Aliquots were tested for viral activity through HA, PA and FFA. The DF-1 cell line was used in two secondary assays where K73164 was incubated for 1hr**.** Aliquots were then taken every 24hrs for the first plate and every 12hrs for the second plate.

Induction of Macrophage-like U937 cells

A previous protocol utilising Phorbol 12-myristate 13-acetate (PMA) to induce a macrophage-like state of the monocyctic cell line, U937. 1000ug of PMA was added to 2mls of DMSO to create a stock solution. The light sensitive stock solution was stored in the dark at -20°C. A 160nm solution of stock PMA in 10% RPMI was used to induce the macrophage-like state (12). The cells remained in this solution for 48 hrs, then were washed and put in fresh 2% RPMI for the duration of the assay. They were then inoculated with virus for 24hrs. Macrophage-like U937 cells at densities of 100,000 cells/ml and 500,000 cells/ml were used for infectivity assays. After 72 hours in non-PMA RPMI a further 160nm PMA in RPMI was added for 48 hrs exposure.

Haemaglutination assay

Haemagglutination assays were performed according to the laboratory standard operating procedure. 10% goose red blood cells were prepared at pH 6.0, as previous work has established K73164 agglutination occurs best at this pH (unpublished data). This solution was then added to serially diluted doubling aliquots (50ul) taken from each time point (t0-7). K73164 antigen was used as a positive control. Negative controls were wells without aliquot added. The plates were read after 45 minutes.

Plaque Assay

Aliquots of 150ul from time 0 to time 7 were diluted into 350ul of blank media and 400ul was inoculated onto a 24 well plate containing a monolayer of Vero cells. After a 1-hour incubation, a methylcellulose overlay was applied to the wells.

Plates were monitored for 4-6 days for plaque formation. If plaques became visible the plate was stained with Methylene Blue to better visualise and count the plaques.

Focus Forming Unit development.

When no obvious plaques were present after the full 7 days**,** the Vero plates were developed to detect Focus Forming Units (FFU). In the case that there were obvious CPE on the cell line plate, a FFA was performed at the end of the 7 days on the plate. The methylcellulose overlay was removed, and the plate was washed twice with phosphate buffered saline (PBS). 100ul of 4% formaldehyde in PBS was added to each well and left for 30-40minutes at room temperature on a plate rocker**,** to fix the cells. Further PBS washes were performed and blocking buffer was added for 1hr. The plates were washed again and 2F2 monoclonal antibody, specific for Chikungunya, Sindbis and Derby virus, was incubated for 1hr (13). Washes were performed and a secondary reporter antibody (Goat Anti-mouse) was incubated for 1hr and then washed off. A pigment substrate; 150ul of True Blue peroxidase, was incubated for 10 minutes to visualise 2F2 binding and the presence of DERV antigen.

RESULTS

Haemagglutination Assays

All plates**,** aside from one**,** had negative results for the presence of red blood cell binding virions. The DF-1 plate with 1hr DERV incubation showed strong haemagglutination from aliquots t1 and t2**,** with a titre of 1024 and 512 respectively. The DF-1 plate with 24hrs DERV incubation showed no haemagglutination.

Plaque Assays and Focus Forming Assays

The SKW cell line produced no plaques or foci in either assay. One large plaque was present in a single duplicate of the Jurkat cell line from the Day 2 aliquot. This did not stain positive with 2F2 for the presence of alphavirus antigen. Induction of the 'macrophage-like state' of PMA exposed U937 cells was determined by morphological change (12). The U937 macrophage-like samples from 100,000 cells/ml density showed up to 5 FFU in the Time 0 aliquots and 1 plaque in the Time 1 aliquots. 5X10^5 cells/ml also produced approximately 10 plaques in Time 0 and less in the Time 1 aliquots. There were no other indicators of viral activity in this cell line.

DERV did not induce CPE in the HeLa cell line for the duration of the assay in comparison to the control wells. HA analysis of samples from all time points showed no indication of viral activity. A FFA performed on the infection plate after t7 detected no presence of alphavirus. A further FFA performed on Vero cells inoculated with t0-t7 aliquots showed many foci from t0 to t3 (24hrs to 96hrs post inoculation) (Figure 2). Most foci were formed in the t0 and t1 aliquot wells. Viral activity decreased after this. Foci in the t2 and t3 aliquots were not well defined.

DERV induced continual mild CPE in the HFF cell line's infection wells for the duration of the infectivity assay (Figure 3). A FFA performed on the HFF infection plate after the infectivity assay showed many foci of infection. The plaque assay of aliquots t0 to t7 (Figure 4) showed many plaques and loss of Vero cells. Most viral activity was seen in the t0 aliquot. Foci of infection, as observed through microscopy, were present in t1. While no plaques were present**,** there was diffuse Vero cell death. Clear plaques were present in t3 and t4. HA tests were all negative for this cell line.

DERV produced high levels of CPE (complete cell lysis) on the DF-1 avian cell line immediately after the 24hr viral incubation period of the infectivity assay. No live cells remained after 3 days, thus the infectivity assay was terminated. The assay was repeated with a viral incubation of 1hr instead of 24hrs, however this assay was also terminated after 3 days

as no live cells remained. In the samples taken from the 1hr incubation plate**,** few plaques were present in the Day 0 aliquot (1hr post infection, immediately after plate wash) and complete cell lysis was present in the Day 1 aliquot (24hrs post infection) (Figure 5). In both plates the highest viral activity was measured at 24hrs post inoculation (t0 in the 24hr incubation plate and t1 in the 1hr incubation plate) (Figure 5). The DF-1 assay was again repeated with 12hr intervals to better capture the short time frame of viral activity. This final DF-1 assay used neat and titrated virus (1:4). Aliquots were taken every 12 hours and the infectivity assay was concluded at 48hrs when no DF-1 cells remained in the infection wells. When the aliquots were tested for viral activity with the plaque assay**,** aliquots from t12 to t48 induced complete Vero cell lysis.

Discussion

DERV tropism is evident in DF-1, HeLa and HFF cell lines, as evidenced by combinations of visible CPE, plaques, focus forming units and haemagglutination. The SKW and Jurkat cell lines, did not appear susceptible and the results of the U937 induced-macrophages suggest they are also not susceptible to infection.

High levels of CPE, plaque formation and a single positive HA result were seen in the DF-1 cell line, suggesting that it is highly susceptible and permissive to DERV infection and replication. Alphaviruses tend to cause obvious CPE in non-mosquito cell lines due to their ability to inhibit host macromolecular synthesis to produce many viral particles (14). For that reason**,** the high titres and obvious CPE observed in the DF-1 cell line is in keeping with previous studies.

DF-1 cells have been previously utilised to study alphavirus replication kinetics. A study using a Sindbis virus cultured in DF-1 cells at 30°C found that virus production increased dramatically after 4 hours**,** then became constant after 6 hours, releasing 2000 plaque forming units per cell per hour (14). If DERV has similar replication kinetics, which is quite likely given its phylogenetic clustering, this would explain the high level of viral activity in many of the t0 aliquots (these were taken after 24hrs incubation), and the low level of viral activity seen in t0 aliquots taken after 1hr incubation (Figure 5). Time 0 aliquots are generally taken to confirm that the washes post infection cleared excess virus and that subsequent plaques from t1-t7 are not leftovers from the initial inoculation. In this experiment, most t0 aliquots showed complete lysis when taken after 24hrs. Strauss' observations of Sindbis replication and this study's observations of complete PA lysis after 24hrs indicate that a period of highly productive viral replication occurred during this 24hr period. Such high titres of virus would not have been effectively cleared after 3 washes. The residual of the high titre virus would then still be present in large enough quantities to cause complete cell lysis in the t0 wells.

The DF-1 avian cell line was one of the more promising cell lines tested**,** as epidemiological data suggested birds as a possible host (4). DERV was isolated during the 2010/2011 MVEV outbreak which coincided with high levels of rainfall/flooding and the highest recorded level of sentinel chicken viraemia (1). MVEV is known to infect bird populations**,** in particular**,** the waterfowls belonging to the *Ciconiiforms* group (8). MVEV outbreaks are also postulated to follow floods and the subsequent bird population increases (15). Therefore**,** given that DERV was isolated at the same time as MVEV**,** both viruses could share a reservoir host. Birds have had a role in the spread of widely distributed alphaviruses such as Sindbis virus (14). As avian cell tropism is a feature of many other alphaviruses, it would not be surprising if DERV followed this pattern. Tropism for birds and a possible avian reservoir host would be important factors contributing to DERV's geographic range and its potential for human infection.

To determine if birds are a potential host for DERV, further cell line tropism studies using other, preferably adult avian cell lines, should be conducted. This is because DF-1 cells are highly susceptible to viral induced cell death, so it is not surprising that such high viral titres and strong CPE were observed (16). Furthermore**,** as embryonic cells are often more susceptible to viral infection due to their increased proliferative potential and cell cycling (17), DF-1 cells may be more susceptible and permissive to viral infection relative to other cell lines (18).

The HeLa cell line showed evidence of DERV susceptibility in the PA and FFA. The t0 aliquots showed viral activity, which would usually imply poor washing. However, given DERV's peak viral production is seemingly within the first 24hrs after inoculation, viral activity in the t0 aliquot is likely of a very high titre. This could cause the appearance of ineffective removal of inoculum as the highly concentrated virus would not be completely removed after 3 washes. Similar viral kinetics have been observed in a study of Chikungunya virus tropism (19). Flow cytometry for viral antigen and TCID50 scores revealed that HeLa cells are susceptive to CHIKV and that 80-100% of cells become infected by 24hrs post infection (19). CHIKV also produced considerable CPE post 24hrs which was not replicated in this study. Although many detached cells were present in the DERV infection wells, this was also present in the control wells and was therefore not considered significant. The FFA performed on the cell line infectivity plate 7 days after DERV inoculation did not show any 2F2 binding or presence of alphavirus. This could be due to the virus having a short period of replication and little antigen remaining after 7 days of media changes or all cells expressing the antigen having died. The result may also be due to human error.

DERV replicated in the Human Foreskin Fibroblast (HFF) cell line; clear CPE and viral production are evidence of this. HFF viral production conforms with the other cell lines tested (Figure 6). However, the t1 (48hrs) well of the PA had a slightly unclear result (Figure 4). It was assumed that this well contained complete cell lysis, as this is similar to the other results, but the methylcellulose used was very loose which could cause plaques to become unclear. HA results cannot corroborate this as the HA for HFF and HeLa cell lines was negative, however, HAs are sensitive to media pH which may cause lack of haemagglutination. DERV infection of a fibroblastic cell line is interesting as it may reflect some of the proposed pathophysiology of Old-World alphaviruses. RRV, SINV and CHIKV can infect connective tissue of the joints and skeletal muscle (20). As DERV is phylogenetically clustered with the arthritogenic alphaviruses, its ability to infect fibroblasts points to a possible arthritogenic pathophysiology. Further testing in muscle or other joint related cell lines would confirm this.

A macrophage cell line was proposed for inclusion in this study as various studies using the closely related RRV have indicated macrophage tropism. RRV antigen has been found to persist in the joint macrophages of RRV patients (21). Previous protocols utilising PMA, have been able to induce the monocyte cell line, U937, into a 'macrophage-like state' (12). U937 cells are not susceptible to DERV, but the PMA induction may change their susceptibility to the virus (unpublished data). The U937 cells in this experiment did achieve the morphological features of a macrophage-like state after PMA exposure. Results from the PA and FFA showed viral activity in the t0 and t1 aliquots. However, unlike most other cell lines exhibiting t0 viral activity, complete cell lysis on a Vero monolayer was not present after 24hrs, and approximately 10-30 plaques and foci were present in the t0 aliquot and tenfold less in the t1. For this reason and in combination with the other t0 results, it is unlikely that any viral replication occurred in the first 24hrs, or that the plaques in t1 are leftovers of the initial inoculation. Macrophage tropism cannot be ruled out by this experiment as the macrophage induction of U937s was not optimised prior to running the

experiment, and the 'induced macrophage model' may not represent all features of *in vivo* macrophages. A protocol utilising PBMC-derived macrophages may prove more successful, as productive infection was observed in the CHIKV tropism study (19). These macrophages may better reflect *in vivo* infection.

Based on this study it is unlikely that DERV infects Jurkat or SKW cell lines, and therefore T and B lymphocytes respectively. Our assays indicated no viral activity and the single unusually large plaque in the Jurkat t2 plaque assay did not bind 2F2, meaning that it was likely an artefact. Animal-model studies of RRV used knock-out (KO) mice to study the involvement of the adaptive immune response in RRV infections. The adaptive immune KO models had identical disease progression to the normal disease model, indicating minimal involvement of adaptive immune cells in RRV disease (21). If RRV or other arthritogenic alphavirus were tropic for T or B cells, it would likely result in a reduction in virulence or immunopathogensis in the KO model (21).

This study of DERV tropism in cell lines has provides the first evidence that DERV may infect human and avian cells. Infection and production of infectious virus was shown in fibroblastic and epithelial cell lines which may point to a pathophysiological role of DERV as one of the arthritogenic alphaviruses. Infection of an avian population is likely given the epidemiological data and DERV's infection of the DF-1 cell line. However, further study in other avian cell lines and supporting seroprevalence data within bird populations would help provide conclusive evidence for an avian reservoir host. A rapid and highly productive replication period was seen across all susceptible cell lines. This validates the high levels of CPE produced after 24hrs and is important to consider when planning future assays that capture DERV's replication time.

FIGURES AND TABLES

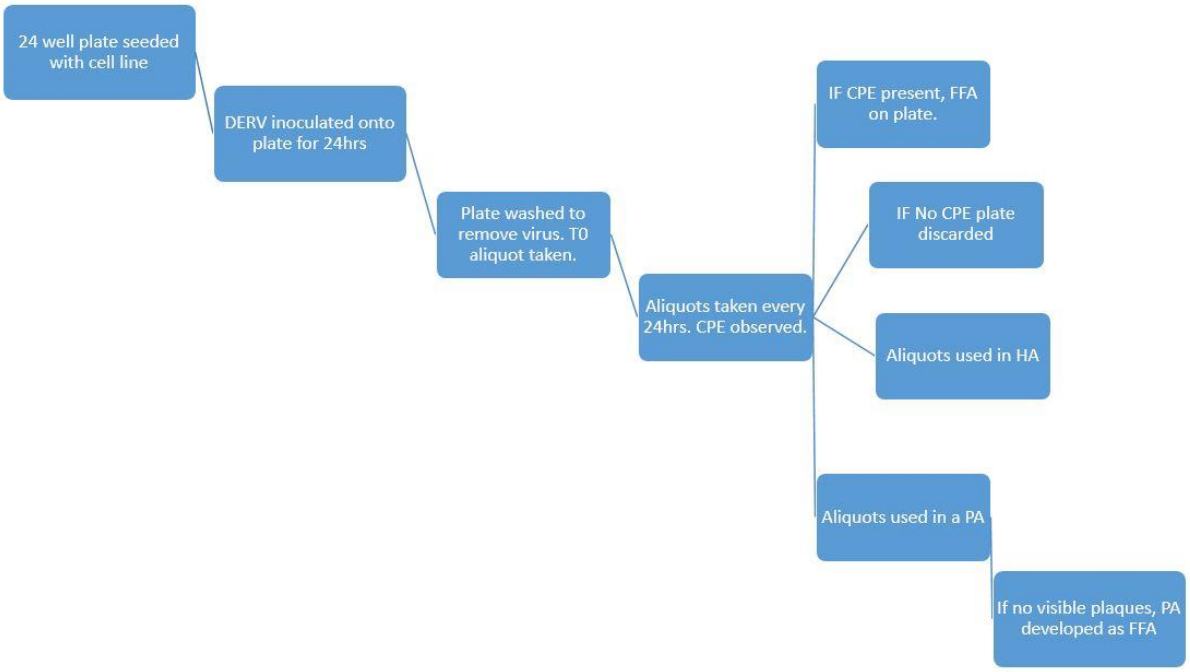

Figure 1: Experimental procedure for each cell line tested. Focus Forming Assay (FFA). Haemagglutination Assay (HA). Plaque Assay (PA).

Cell Line	Type	adherent /suspension	culture media	Assays
SKW	B Lymphocyte, EBV transformed	suspension	5% RPMI	1
JURKAT	T Lymphocyte,	suspension	5% RPMI	1
U937 PMA,	monocyte derived macrophage	adherent	10% RPMI	2
Hela	Cervical epithelial cell	adherent	10% DMEM	2
DF-1	Avian embryonic fibroblast	adherent	10% DMEN	3
HFF	Foreskin fibroblast	adherent	10% DMEN	1

Table 1: Cell lines tested in this experiment. Assays = the number of assays they were used for.

Cell Line	CPE	HA	PA	Vero FFA	Cell Line FFA
SKW	-	-	-	-	NA
JURKAT	-	-	+	-	NA
U937 PMA, 1x10^4c/ml	-	-	-	+	NA
U937 PMA, 5x10^4c/ml	-	-	+	-	NA
Hela 1	-	-	+++	NA	NA
Hela 2	-	-	NA	+++	-
DF-1 24hr incubation	+++	-	+++	NA	NA
DF-1 1hr incubation	+++	+++	+++	NA	NA
DF-1 1hr incubation Neat, 12hr	+++	NA	+++	NA	NA
DF-1 1hr incubation 4^1, 12hr	+++	NA	+++	NA	NA
HFF	++	-	++	NA	++

Table: 2. Cell line summary. Results of all cell lines tested. +++ strong positive (100+ plaques of focus forming units or Haemagglutination). ++ weak positive (50-100 plaques) + weaker positive (0-10 plaques). – (no plaques or haemagglutination). NA = not assessed. Hela cell line was assayed twice as the t0 aliquots were missing.

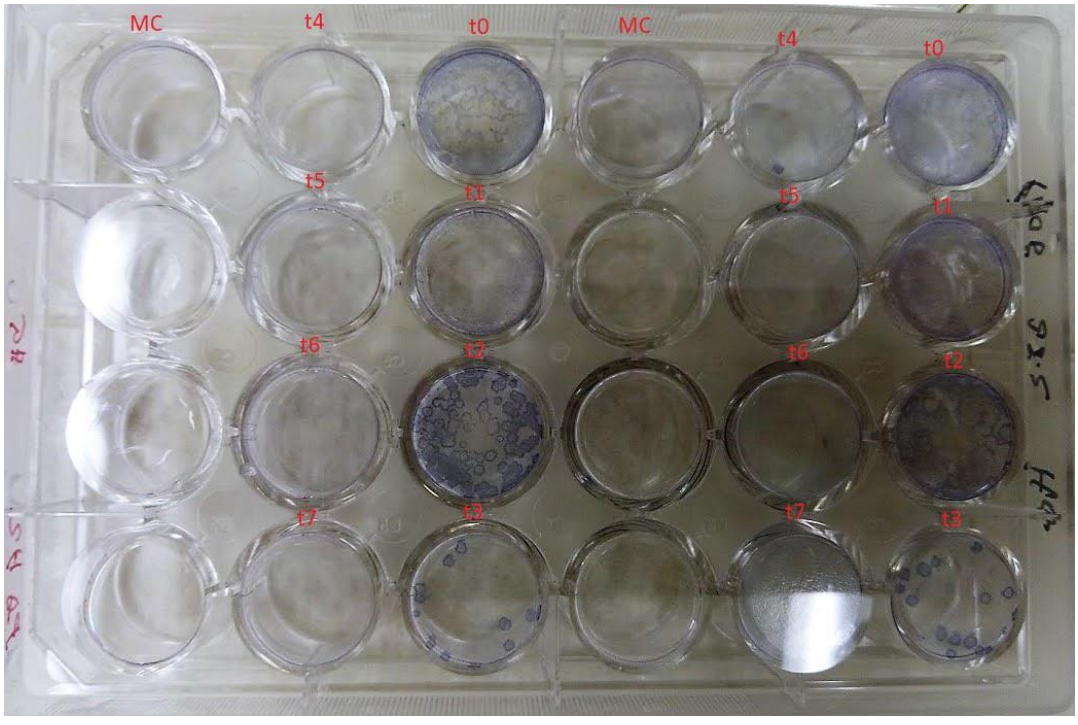

Figure: 3. Focus Forming Assay using 2F2 on a Vero monolayer with aliquots from the HeLa DERV infection plate.

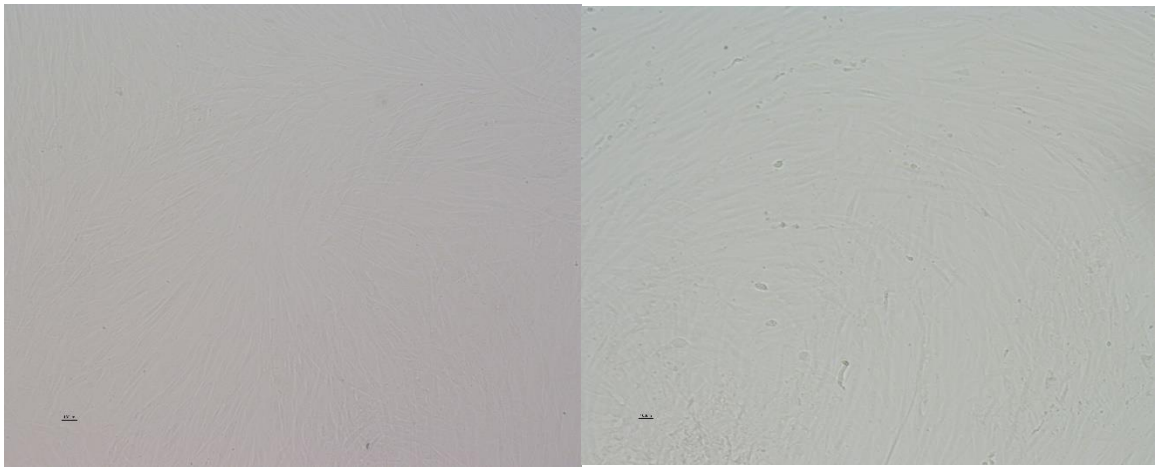

Figure: 4. t0 (24hr post DERV inoculation) of infection and control wells. Rounded and granular cells are present in the infection well.

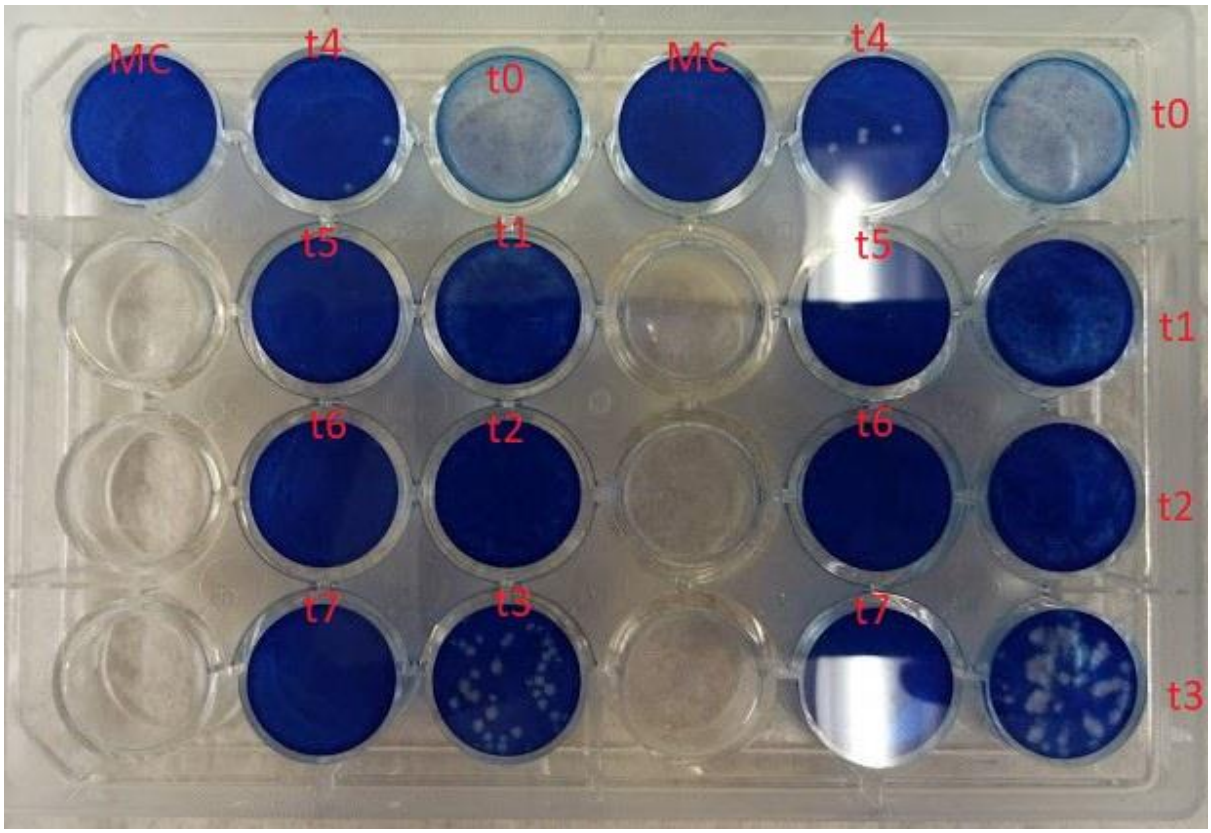

Figure: 5. HFF Plaque Assay on Vero Monolayer.

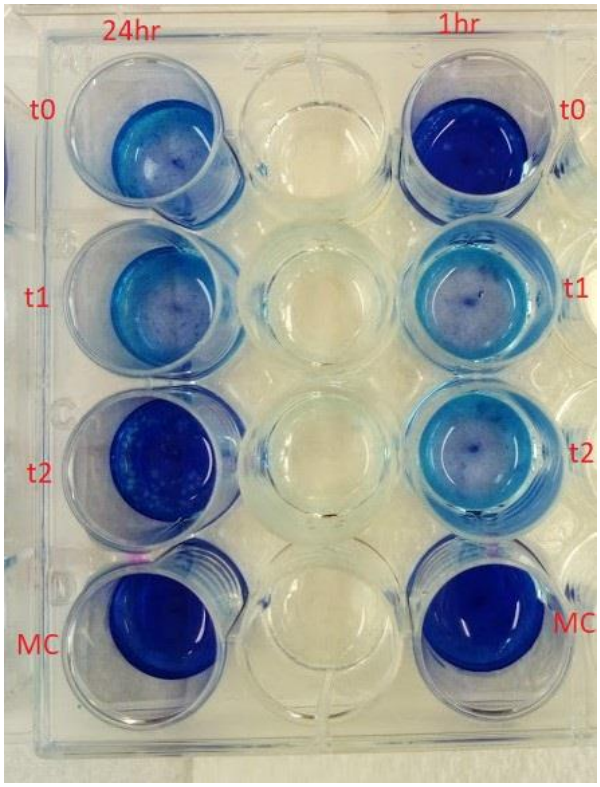

Figure: 6. DF-1 Plaque Assay, aliquots from the 24hr incubation (left) and aliquots from the 1hr incubation (right).

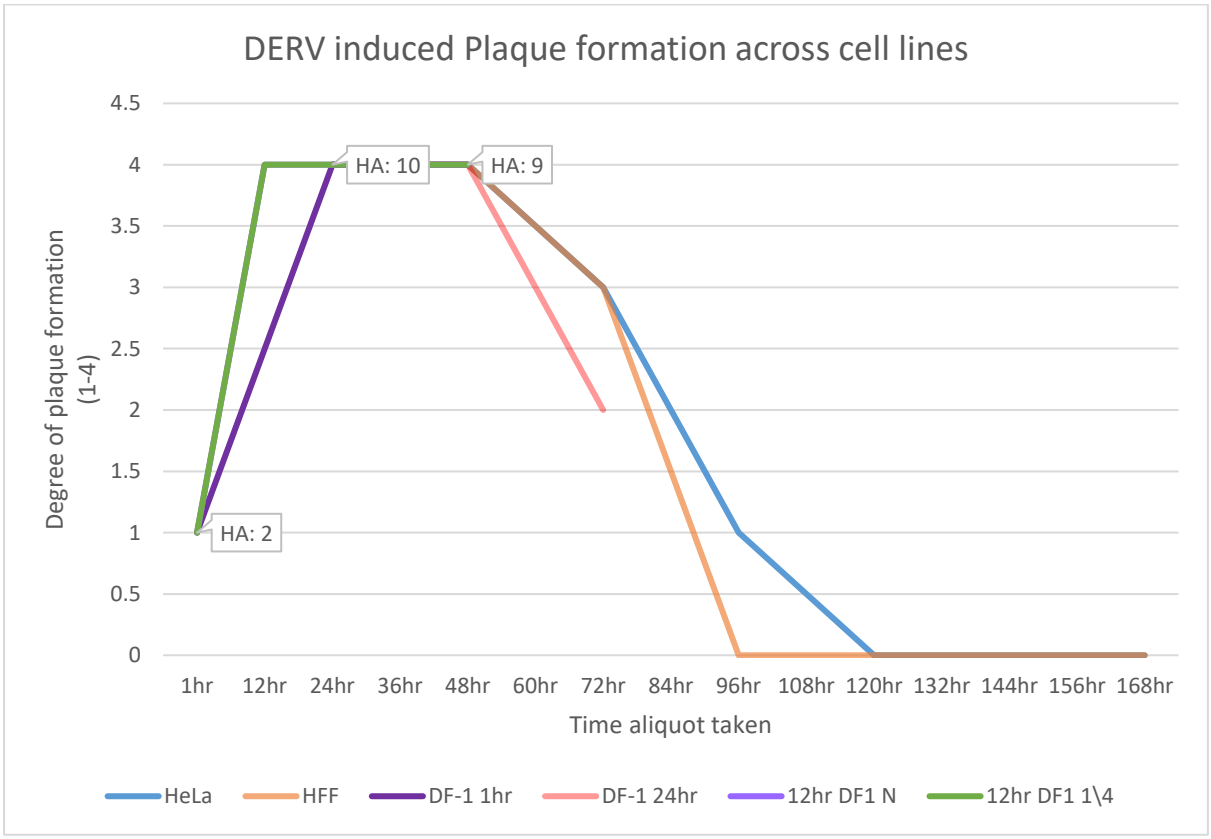

Figure: 7. Summary of plaque assays of cell lines with viral activity. Plaque formation is measured on a score of 1-4, with 1 being 1-20 plaques and 4 being complete cell lysis.

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