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Research article

Sucrose density gradient centrifugation and cross-flow filtration methods for the production of arbovirus antigens inactivated by binary ethylenimine

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Abstract

Background: Sucrose density gradient centrifugation and cross-flow filtration methods have been developed and standardised for the safe and reproducible production of inactivated arbovirus antigens which are appropriate for use in diagnostic serological applications.

Methods: To optimise the maximum titre of growth during the propagation of arboviruses, the multiplicity of infection and choice of cell line were investigated using stocks of Ross River virus and Barmah Forest virus grown in both mosquito and mammalian cell lines. To standardise and improve the efficacy of the inactivation of arboviral suspensions, stocks of Ross River virus, Barmah Forest virus, Japanese encephalitis virus, Murray Valley encephalitis virus and Alfuy virus were chemically inactivated using binary ethylenimine at a final concentration of 3 mM. Aliquots were then taken at hourly intervals and crude inactivation rates were determined for each virus using a plaque assay. To ensure complete inactivation, the same aliquots were each passaged 3 times in *Aedes albopictus* C6/36 cells and the presence of viral growth was detected using an immunofluorescent assay. For larger quantities of viral suspensions, centrifugation on an isopycnic sucrose density gradient or cross-flow filtration was used to produce concentrated, pure antigens or partially concentrated, semi-purified antigens respectively.

Results: The results of the propagation experiments suggested that the maximum viral titres obtained for both Ross River virus and Barmah Forest virus were affected by the incubation period and choice of cell line, rather than the use of different multiplicity of infection values. Results of the binary ethylenimine inactivation trial suggested that standardised periods of 5 or 8 hours would be suitable to ensure effective and complete inactivation for a number of different arboviral antigens.

Conclusion: Two methods used to prepare inactivated arbovirus antigens have been standardised to minimise production failure and expenditure and to provide reagents that conform to the highest quality and safety requirements of a diagnostic serology laboratory. The antigens are suitable for use in either enzyme linked immunosorbent assays or haemagglutination inhibition assays and the optimised protocols can be directly applied to produce antigens from new or emerging arboviral pathogens.

Background

Arthropod-borne viruses (arboviruses) may be transmitted to man and other susceptible vertebrate hosts by infected arthropods such as mosquitoes. There are over 100 arboviruses which are known to cause human infections with varying degrees of morbidity and mortality [1]. Included in the arbovirus group are single-stranded, positive sense RNA viruses belonging to the *Alphavirus* and *Flavivirus* genera of the families *Togaviridae* and *Flaviviridae* respectively [2]. In Australia, the alphaviruses that have been implicated in human disease include Ross River virus (RRV), Barmah Forest virus (BFV) and Sindbis virus, whilst flaviviruses include Dengue virus serotypes 1-4 (DENV 1-4), Murray Valley encephalitis virus (MVEV), Kunjin virus (KUNV), Japanese encephalitis virus (JEV) and Kokobera virus. Additionally, antibodies which react with 2 other flaviviruses namely, Alfuy virus (ALFV) and Stratford virus, have also been found in human sera.

The serological diagnosis of arbovirus infections usually involves the testing of paired acute and convalescent sera in parallel, and requires stable, reliable antigens that are reproducible and provide consistent results. The development of standardised protocols to ensure the availability of diagnostic antigens is an essential requirement in any arboviral laboratory, and allows the testing of human and animal sentinel surveillance sera for the diagnosis and control of arboviral diseases. In addition, production of antigens that are suitable for multiple serological assays such as the enzyme linked immunosorbent assay (ELISA) and the haemagglutination inhibition (HAI) assay, improves the efficiency of the diagnostic laboratory and increases the accuracy, reproducibility and reliability of the results produced.

The production of antigens requires the propagation of large quantities of high titre arboviral suspensions in cell culture. Parameters such as choice of cell line, multiplicity of infection (m.o.i.) and length of incubation period, should be considered to obtain optimal viral growth. The formation of nonviable deletion mutants or defective interfering (DI) particles, a well known phenomenon resulting from serial, high multiplicity passaging of many viruses in cell culture, can interfere with the replication of infectious virus and reduce yields [3-5]. To reduce the effects of DI particle formation during the propagation of virus, it may be useful to infect a particular cell line with a stock virus previously grown in a different cell line.

Methods to produce antigens may vary depending upon the amount and purity of antigen required and protocols usually involve four steps: clarification by centrifugation, inactivation, concentration and purification. Inactivation of viruses during antigen production is not only essential to prevent cross-contamination, but is also implemented to minimise the exposure of personnel to biological hazards and maintain a high standard of safe laboratory practices and procedures.

Binary ethylenimine (BEI) has been used to successfully inactivate a number of RNA and DNA animal viruses for antigen and vaccine production, including foot-and-mouth disease virus [6], Rabies virus [7] and Newcastle disease virus [8]. Although potentially carcinogenic, use of a stock solution of 0.1 M BEI is far less toxic [9] and as a chemical inactivant, may be more effective than other means of inactivation such as ultraviolet radiation [10]. BEI is known to act on nucleic acid, with minimal effect on the immunogenic properties of the viral protein coat. In addition, we have chosen BEI inactivation as the reagents required are inexpensive and the antigens produced are highly stable [7].

Two methods for concentrating and purifying inactivated arboviral antigens, namely, the sucrose density gradient centrifugation (SDGC) method and the cross-flow filtration (CFF) method, were investigated. Viruses have been purified previously by layering preparations on an isopycnic (equilibrium) sucrose density gradient [11]. A similar method using a potassium tartrate gradient has been used to prepare a purified RRV antigen for use in an IgM ELISA [12].

Cross-flow filtration technology has been utilized for many applications including the purification of water [13] and immunoglobulins [14]. In comparison to conventional filtration methods, the use of CFF to concentrate viral particles has several advantages. During CFF, culture fluid is re-circulated in tangential flow, parallel to the filter membrane. Build-up of viral particles on the membrane is minimised by the recirculation of fluid over the surface, which also facilitates the concentration of particles present in the retentate fluid. In this way, alphavirus and flavivirus particles may be partially concentrated and purified from culture fluids with the use of an appropriately sized filter membrane.

The production of substantial quantities of good quality, adequately concentrated antigens is crucial for the reliable performance of diagnostic, serological assays. We describe standardised, reproducible methods to produce safe, inactivated arboviral antigens for use in serological tests using either SDGC or CFF protocols. The methods described in this paper produce inactivated reagents that can be used immediately in a variety of ELISA and HAI assays, decreasing hazardous risks to laboratory personnel and the requirement of multiple protocols to produce different antigens. In Australia, the application of these methods for the production of antigens to exotic and emerging arboviruses such as Chikungunya virus (CHIKV), West

Table 1: Optimal m.o.i. values using RRV C6/36 stock virus with respective HA titre and hours of incubation in C6/36, PS-EK, Vero and BHK-21 C_{13} cell lines

Cell Line	m.o.i. (PFU/cell)	Maximum HA Titre at pH 5.8	Hours of Incubation
C6/36	1.5×10^{-4}	1024	51
	1.5×10^{-5}	1024	51
PS-EK	1.1×10^{-1}	1024	47
	4.4×10^{-2}	1024	47
	2.2×10^{-2}	1024	51
Vero	7.5×10^{-2}	8192	47
	3.0×10^{-2}	8192	47
	1.5×10^{-2}	8192	47
	1.5×10^{-3}	8192	47
	1.5×10^{-4}	8192	47
BHK-21 C_{13}	7.9×10^{-2}	8192	51
	3.2×10^{-2}	8192	47
	1.6×10^{-2}	8192	47
	1.6×10^{-3}	8192	51

Nile virus (WNV) and Yellow fever virus (YFV), enables the development of diagnostic assays for the future identification, management and control of outbreaks caused by these pathogens.

Results

Optimal m.o.i. and choice of cell line

The haemagglutination (HA) titres (pH 5.8) obtained from the optimal m.o.i. experiments using the RRV C6/36 stock were plotted against the hours of incubation for each cell line infected (data not shown). Haemagglutination titres peaked by 47 hours post infection (Table 1). The highest HA titre obtained was 8192, resulting from the infection of Vero or BHK-21 C_{13} cells using m.o.i. values of approximately 8.0×10^{-2} to 2.0×10^{-4} plaque forming units per cell (PFU/cell) and 8.0×10^{-2} to 2.0×10^{-3} PFU/cell respectively. In comparison, the maximum HA titre that could be obtained from the infection of C6/36 or a clone of porcine stable (PS-EK) cells [15] was 1024. HA titres were not obtained from the negative control flasks.

Results from the BFV C6/36 stock optimum m.o.i. trials demonstrated that viral HA titres (pH 5.8) peaked by 69 hours post infection (data not shown). The highest HA titre that could be obtained from the infection of either C6/36 or Vero cells, was 4096, using m.o.i. values of 1.5×10^{-1} to 7.5×10^{-5} PFU/cell. The BH2193 strain of BFV used could not be propagated in PS-EK cells.

BEI virus inactivation

The results of the alphavirus BEI inactivation trial using the RRV and BFV C6/36 virus stocks are presented in Figure 1. The RRV C6/36 virus stock containing 2.0×10^7 plaque forming units per millilitre (PFU/mL) was inactivated in 3 hours at 37°C, which can be expressed as an inactivation rate of 2.4 Log_{10} PFU/mL of virus per hour (Log_{10} PFU/mL.h^{-1}). Similarly, the BFV C6/36 virus stock containing 1.0×10^8 PFU/mL was inactivated in 4 hours at 37°C which produced an inactivation rate of 2.0 Log_{10} PFU/mL.h^{-1}.

The results of the flavivirus BEI inactivation trial using the JEV and MVEV C6/36 virus stocks and the ALFV PS-EK virus stock are presented in Figure 2. A summary of the results from both the alphavirus and flavivirus inactivation trials using a plaque assay are given in Table 2.

The time required for complete inactivation of the RRV C6/36 virus stock samples was determined to be 3 hours by plaque assay and 4 hours by immunofluorescent assay (IFA) after serial passage in C6/36 cells. Similarly, the time required to completely inactivate the JEV C6/36 and the ALFV PS-EK virus stock samples was determined by serial passage and IFA to be an hour longer than the time determined by plaque assay (data not shown). The time required for complete inactivation of the DENV-1 C6/36 virus stock samples was determined by IFA to be 2 hours following 3 passages in C6/36 cells.

Antigen production

Details of the inactivated arbovirus antigens made using either the SDGC or CFF methods are listed in Table 3. The inactivation times used were at least twice the number of hours determined from the BEI inactivation trial experiments. For the CHIKV antigen, 8 hours was used as the inactivation time based on the results of the other alphaviruses, RRV and BFV. For the flaviviruses WNV, JEV, MVEV, KUNV, ALFV, YFV and DENV1-4, antigens were initally inactivated for 5 hours based on the results from the flavivirus inactivation trial. All antigens were successfully inactivated, except DENV-2 and MVEV, for which the inactivation time had to be increased to 8 hours. Complete inactivation of all antigens was confirmed by IFA and the absence of viral growth following serial passage in C6/36 cells.

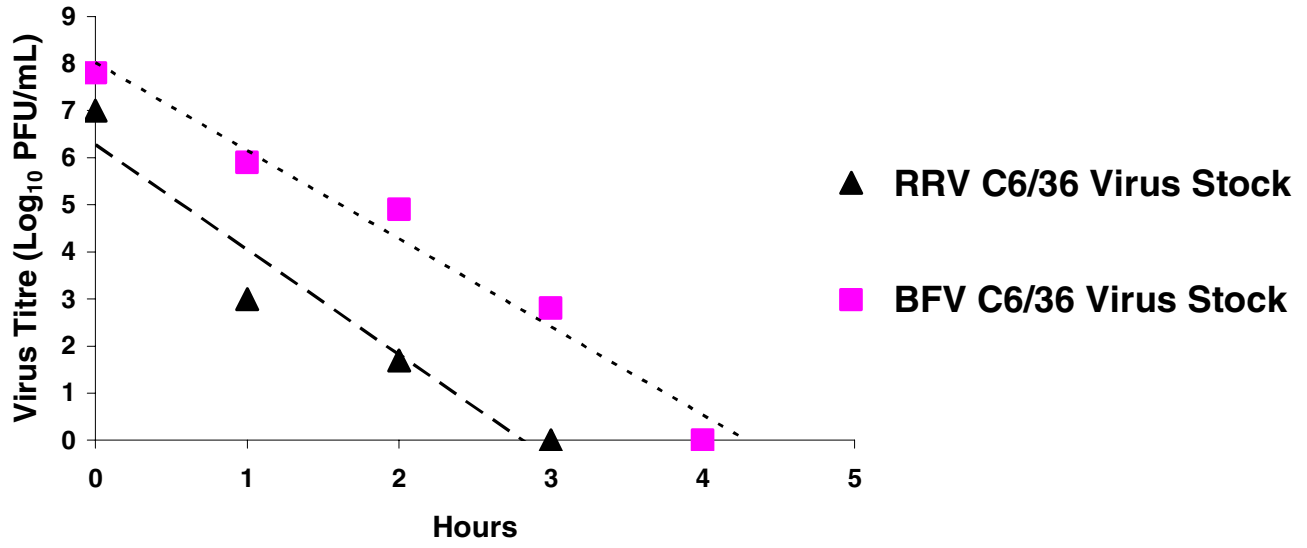

Figure 1
BEI inactivation of RRV and BFV C6/36 virus stocks at 37°C. The viral titres (Log_{10} PFU/mL) are shown decreasing with time (hours).

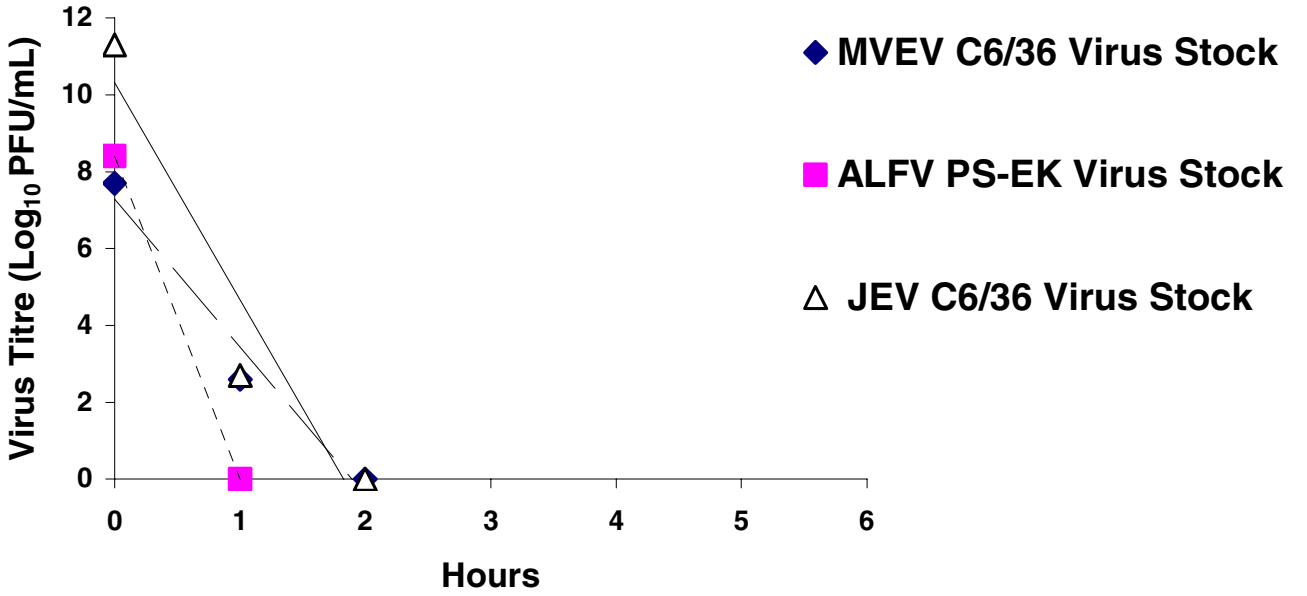

Figure 2
BEI inactivation of the JEV and MVEV C6/36 virus stocks and of the ALFV PS-EK virus stock at 37°C. The viral titres (Log_{10}PFU/mL) are shown decreasing with time (hours).

Table 2: Results of BEI alphavirus and flavivirus BEI inactivation trials at 37°C

Virus Stock	Strain	PFU/mL	Hours for Complete BEI Inactivation	Inactivation Rate (Log_{10} PFU/mL.h^{-1})
RRV C6/36	T48	2.0×10^7	3	2.4
BFV C6/36	BH2193	1.0×10^8	4	2.0
JEV C6/36	TS00	2.1×10^{11}	1	11.3
MVEV C6/36	MRM66	2.5×10^8	2	4.2
ALFV PS-EK	MRM3926	1.0×10^8	1	8.0

Table 3: Details of virus stocks, cell lines and BEI inactivated arbovirus antigens

Virus Stock	Strain	Cell Line	Incubation (days/°C)	HA upon Harvest (titre/pH)	Inactivation Time (Hours)	Antigen Method
RRV C6/36	T48	Vero	3/37	2048/5.8	8	CFF/SDGC
BFV C6/36	BH2193	Vero	3/37	2048/5.8	8	CFF/SDGC
CHIKV MBP	BKM459	C6/36	2/28	1024/5.8	8	CFF
JEV PS-EK	Nakayama	PS-EK	3/37	2048/6.2	5	CFF/SDGC
KUNV C6/36	MRM16	PS-EK	3/37	1024/6.4	5	CFF
MVEV C6/36	MRM66	PS-EK	3/37	2048/6.4	8	CFF/SDGC
ALFV C6/36	MRM3926	PS-EK	3/37	512/6.4	5	CFF
DENV-1 C6/36	TIM00	C6/36	13/28	256/6.2	5	CFF
DENV-2 C6/36	TIM00	C6/36	8/28	256/6.2	8	CFF/SDGC
DENV-3 C6/36	TIM00	C6/36	7/28	512/6.2	5	CFF/SDGC
DENV-4 C6/36	TIM00	C6/36	11/28	256/6.2	5	CFF/SDGC
WNV C6/36	Sarafend	PS-EK	2/37	1024/6.2	5	CFF
YFV C6/36	17D	PS-EK	3/37	128/6.4	5	CFF

Discussion

The optimisation of viral growth to obtain maximum viral titres is necessary to produce high quality arboviral antigens. In particular, our results suggested that the choice of cell line may be more important than determining the optimal m.o.i. when propagating arboviruses for antigen production. Results of the optimal m.o.i. experiment using RRV, demonstrated that at 47 hours post infection, a maximum HA titre of 8192 could be produced in either Vero or BHK-21 C_{13} vertebrate cells using the C6/36 virus stock. This titre was obtained using several m.o.i. values which varied as much as 2.0 Log_{10} PFU/cell. In contrast, a lower maximum HA titre of 1024 resulted from the infection of PS-EK cells which was identical to the maximum HA titre produced from the infection of C6/36 cells. Although the use of C6/36 cells has been recommended previously for the purpose of propagating RRV easily and cheaply for use in antigen production [12], our results suggested that RRV could be grown to a higher titre in Vero or BHK-21 C_{13} cells efficiently with comparable expense and use of resources.

In comparison, results from the optimal m.o.i. experiment using BFV demonstrated that at 69 hours post infection, virus could be grown to a maximum HA titre of 4096 by infecting either C6/36 or Vero cells with the C6/36 virus stock. Similarly, several different m.o.i. values, which varied as much as 5.0 Log_{10} PFU/cell, were used to produce the same high HA titre. These results demonstrated that for a given arbovirus stock, several m.o.i. values may be used to produce the same maximum HA titre, provided the appropriate choice of cell line and incubation period is chosen. The use of higher virus dilutions also did not improve the maximum virus HA titres of 8192 and 4096 obtained in the RRV and BFV m.o.i. experiments respectively.

The results from the BEI inactivation experiments demonstrated that the lowest BEI inactivation rates (Log_{10} PFU/mL.h^{-1}) were obtained for RRV and BFV alphavirus stocks, whilst higher inactivation rates were obtained for JEV, MVEV and ALFV flavivirus stocks. Factors affecting BEI inactivation rates have been extensively discussed elsewhere [6,9]. The inactivation rates were calculated using the results from the plaque assays. Interestingly, when the rates for the RRV, JEV and ALFV stocks were recalculated based on the C6/36 cell culture results (data not shown), higher inactivation rates were still achieved for the flavivirus stocks, 2 of which had similar PFU/mL values to that of the alphavirus stocks (Table 2). In addition to the determination of inactivation rates, we have also demonstrated

the ability to inactivate a range of both alphaviruses and flaviviruses efficiently.

Effective virus inactivation methods should achieve the irreversible loss of viral infectivity, with minimal loss of antigenic properties [7,10]. Previous experiments have suggested the use of 3 mM BEI and an incubation period of 24 hours to inactivate a RRV Vero virus stock for vaccine production [16]. However, in our experiments, 3 mM BEI was used to inactivate more than 7.0 Log_{10} PFU/mL of a RRV C6/36 virus stock in 4 hours. Although the results of the IFA experiments following serial passage in C6/36 cells suggested 4 hours, we extended the BEI incubation time to 8 hours and have been able to successfully make safe and stable RRV antigens repeatedly. Indeed, passaging 3 times in cell culture following inactivation was not only found to be more sensitive than plaque assay for detecting residual RRV, but also for detecting residual JEV and ALFV.

Whilst BEI inactivation rates were determined for the recent Australian JEV strain TS00 [17], we chose virus stocks of the Nakayama vaccine strain of JEV as a safer alternative for the production of large volumes of diagnostic antigen. The same propagation and inactivation conditions used for the TS00 JEV strain were applied, and the resulting Nakayama JEV strain antigen has been used successfully in ELISA and HAI assays to detect seroconversions in sentinel pigs [17-19].

We have now successfully produced arbovirus antigens inactivated by 3 mM BEI using isopycnic SDGC or CFF methods, including antigens of 3 viruses exotic to Australia, namely, CHIKV, YFV and WNV. The Sarafend strain of WNV was used to make an antigen as it was the only strain initially available for this study. Recently, the NY99 strain of WNV has been obtained and a diagnostic antigen using this strain will be produced. However it has been shown previously that the amino acid homology between the NY99 WNV and KUNV is 98–99% [20] and it is therefore possible that sera obtained from patients infected with NY99 WNV, may demonstrate cross reactive antibodies against the KUNV antigen.

Although the repeatability of inactivation rates was not investigated, the inactivation period for the RRV, BFV, JEV, MVEV and ALFV stocks was extended so that it became least twice the time interval previously determined for each virus. To provide a standardised protocol, 8 and 5 hours were determined to be adequate inactivation times for alphaviruses and flaviviruses respectively. However in comparison to other flaviviruses, when large volumes of MVEV and DENV-2 antigens were produced, it became necessary to extend the BEI incubation time from 5 to 8 hours to achieve complete inactivation. Further investigations of the pH and osmolarity of antigen suspensions, which are known to affect the rate of BEI inactivation [9], may provide a possible explanation for the difference observed in inactivation rates. However, BEI inactivation for 5 or 8 hours has proven repeatedly successful in the inactivation of all the arbovirus antigens grown from the virus stocks listed in Table 3 and provides a standardised protocol for different virus cell culture preparations that may vary significantly in HA titre. Furthermore, to ensure complete inactivation, each antigen is routinely passaged 3 times in C6/36 cells to ensure complete inactivation.

Conclusions

The accurate and timely serological diagnosis of arboviral infections requires the propagation, inactivation, purification and concentration of suitable antigens that can be produced efficiently, safely and reliably. We have demonstrated that the inactivated arbovirus antigens produced by SDGC or CFF protocols, are safe, high quality reagents suitable for serological applications in the diagnostic laboratory. The abilitity to use these antigens in more than one serological assay has also enabled the screening of human or sentinel animal sera against the same antigens. This has improved the efficiency of diagnosis and accuracy of results required for the identification, management and control of outbreaks. In particular, seroconversions to JEV and DENV infections during several recent outbreaks in Australia, were detected using antigens that we have produced by these methods in ELISA and HAI assays [17-19,21-23]. Furthermore, the availability of CHIKV and YFV antigens may assist in the prevention of future outbreaks of these exotic pathogens within Australia, whilst a method to produce a safe NY99 WNV antigen would be of particular importance to countries in North America currently experiencing outbreaks of this strain of WNV. The establishment of improved, standardised methods for the production of inactivated arbovirus antigens has also reduced production failure and expenditure and significantly decreased exposure of laboratory personnel to hazardous reagents.

Methods

All centrifugation procedures were performed using a Beckman Ultracentrifuge Optima L-80 (Beckman Instruments, Inc., Fullerton, California) at 4°C.

Virus stocks

Viruses were propagated in C6/36 (American Type Culture Collection (ATCC) number CRL-1660), PS-EK cells, Vero (ATCC number CCL-81) or BHK-21 C_{13} (ATCC number CCL-10) cells (Table 3). Briefly, each virus was diluted in a maintenance medium consisting of RPMI 1640 (CSL Limited, Parkville, VIC, Australia), 1% Hepes (Gibco BRL® Invitrogen Pty, Ltd, Melbourne, VIC, Australia) and 0.2% bovine serum albumin Fraction V (Gibco

BRL® Invitrogen Pty, Ltd, Melbourne, VIC, Australia). The diluted virus was then used to inoculate confluent cell monolayers grown in 10 × 180 cm^2 flasks or 1 × expanded surface 1700 cm^2 roller bottle (Corning Costar Corporation, Cambridge, MA, USA). Following incubation for 1 hour at either 28°C (C6/36 cells) or 37°C (PS-EK, Vero or BHK-21 C_{13} cells), cultures were re-fed with the same maintenance medium (30 mLs per flask or 300 mLs per roller bottle). After an appropriate incubation period, the cell culture supernatant was harvested and the viral HA titre was determined using a modification of the method used by Clarke and Casals [24]. All subsequent virus dilutions, inoculations and HA titre determinations were performed under similar conditions.

Optimal m.o.i. and choice of cell line

The RRV C6/36 stock was used to investigate the optimal m.o.i.and appropriate cell line required to obtain a maximum viral titre following inoculation. Briefly, confluent monolayers of C6/36, PS-EK, Vero and BHK-21 C_{13} cells (9.9×10^6, 6.6×10^6, 1.0×10^7 and 9.5×10^6 cells per 25 cm^2 flask respectively) were infected with 6 dilutions of virus. This resulted in m.o.i. values of 7.5×10^{-2} to 2.2×10^{-5} PFU/cell. A negative control flask was also included in each experiment. Following 22 hours post infection, an aliquot of 50 μL of culture supernatant from each flask was taken at appropriate intervals for the quantification of HA titres at pH 5.8.

Similar experiments were also performed for the BFV C6/36 stock, which was used to infect C6/36, Vero and PS-EK cells with m.o.i. values of 4.0×10^{-1} to 8.0×10^{-5} PFU/cell.

BEI virus inactivation

To determine the appropriate time required to inactivate RRV virus antigens with BEI, inactivation trials using the RRV C6/36 virus stock were performed using a modification of the method used by Larghi and Nebel [7] and 2-bromoethylamine (Sigma Chemical Co., St. Louis, MO, USA). Briefly, a 0.1 M BEI solution was prepared in 0.175 N sodium hydroxide, filtered through a 0.22 μm filter (Millipore Corporation, Bedford, MA 01730, USA) and incubated at 37°C in a waterbath for 30 minutes (final pH 8.5). An aliquot of 152 μL was added to 5 mLs of the virus stock (final concentration of 3 mM BEI) and the virus suspension was incubated at 37°C using a waterbath. Samples of 250 μL were taken at hourly intervals between 0 and 6 hours and also at 24 hours, before the inactivation reaction was stopped with 25 μL of cold 1 M sodium thiosulphate. A modification of the method used by Gorman and others was used to determine the PFU/mL of the original virus stock and subsequent inactivated samples [15]. A carboxy-methyl cellulose overlay medium was used and plaques were stained with naphthalene black [25]. An aliquot of each inactivated sample was also passaged 3 times in C6/36 cells and the presence of any virus growth was determined using a panel of monoclonal antibodies in an IFA. Similarly, inactivation trials were also performed for BFV, MVEV, JEV and ALFV virus stocks. The DENV-1 C6/36 virus stock could not be plaqued, so inactivation was only determined from the assessment of virus viability by IFA after serial passage in C6/36 cells.

Viral antigen growth and antigen inactivation

Larger quantities of arboviruses were grown in cell culture for antigen production, including 3 viruses exotic to Australia, namely, CHIKV, WNV and YFV. For propagation of viruses in C6/36 cells, 30 × 180 cm^2 flasks (7.1×10^7 cells and 3 mLs of inoculum per flask) were used, whilst for viruses grown in Vero or PS-EK cells, 3 × 1700 cm^2 roller bottles (6.8×10^8 and 4.4×10^8 cells and 30 mLs of inoculum per bottle respectively) were used. The cell culture supernatant (approximately 900 mLs) was harvested once the HA titre had peaked, clarified by centrifugation at 10 000 × *g* for 30 minutes, and filtered through a sterile vacuum-driven 0.22 μm filter (Millipore Corporation, Bedford, MA 01730, USA). A 0.1 M solution of BEI was prepared as above in 27.3 mLs of 0.175 N sodium hydroxide, and added to the filtered virus suspension. Inactivation was performed for 5 or 8 hours, depending on the determined optimal inactivation time, and the reaction was stopped by addition of 90 mLs of cold 1 M sodium thiosulphate. The inactivated virus suspension was stored at 4°C overnight if antigen processing could not be performed immediately.

Antigen production

Sucrose density gradient centrifugation

To pellet the virus, the inactivated virus suspension was centrifuged at 10 000 × *g* for 17 hours overnight. A 5–50% (w/v) isopycnic sucrose density gradient was made in a Beckman 25 × 76 mm Ultraclear tube (Beckman Instruments, Inc., Fullerton, California) using a modification of the method described by Brakke [11]. Briefly, the virus pellet was resuspended in 1 to 2 mLs of cold borate-saline buffer pH 9.0 [24], carefully layered onto the sucrose gradient and then centrifuged at 85 000 × *g* for 2 hours and 30 minutes. The virus band was harvested using a sterile Pasteur pipette, transferred to a Beckman 3 × 51 mm polyallomer tube (Beckman Instruments, Inc., Fullerton, California) and diluted with cold borate-saline pH 9.0, almost filling the tube. The purified virus was collected by centrifugation at 240 000 × *g* for 1 hour and 30 minutes, and the virus pellet was resuspended in 0.5 to 1.0 mL of cold borate-saline pH 9.0. Inactivation of the concentrated purifed virus antigen was checked by filtering a small aliquot (1:20 dilution) through a 0.22 μm filter and passaging 3 times in C6/36 cells as described above. The viral antigen was stored at -80°C.

Antigen production

Cross-flow filtration

A CFF system consisting of a Satorius Sartocon Mini SM 17521 flow-distributing holding device, filter unit SM 3031466901E and peristaltic pump SM 16650 (Sartorius AG, Goettingen, Germany) was used to prepare partially concentrated, semi-purified viral antigens. Briefly, the BEI inactivated viral suspension was recirculated parallel to the filter unit (100,000 nomimal molecular weight cutoff) in tangential flow with the aid of the peristaltic pump under minimal pressure (<1 bar) until the volume of the retentate was reduced to approximately 20 mLs. The inlet tube was removed from the bottle containing the virus suspension and the pumping resumed until as much of the virus suspension as possible was recovered (approximately 100 mLs). This resulted in a ten-fold concentration of the original virus suspension.

The pH of the virus antigen was adjusted to pH 9.0 by addition of approximately 10 to 20 mLs of 0.25 M Tris-HCl (pH 10.0). An aliquot was taken to determine the post concentration HA titre and also to perform an inactivation check as described for the SDGC antigen method. The viral antigen was stored at -80°C.

Competing interests

There are no competing interests associated with this study.

Authors' contributions

AP performed growth of virus stocks, all experiments, evaluations and production of antigens. TC assisted in the growth of virus stocks and BEI inactivation trial experiments. DP assisted in the design and implementation of the protocols used for the sucrose density gradient centrifugation and cross-flow filtration of arbovirus antigens. GS assisted in the design of m.o.i. and inactivation trial experiments for the optimisation and standarisation of the growth and inactivation of viral suspensions used for antigen production. All authors have read and approved the final manuscript.

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