Virus-specific thermostability and heat inactivation profiles of alphaviruses

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Serological diagnosis is a critical component for disease surveillance and is important to address the increase in incidence and disease burden of alphaviruses, such as the chikungunya (CHIKV) and Ross River (RRV) viruses. The gold standard for serological diagnosis is the plaque reduction neutralization test (PRNT), which demonstrates the neutralizing capacity of serum samples after the removal of complement activity and adventitious viruses. This procedure is normally performed following inactivation of the virus at 56 °C for 30 min. Although this protocol has been widely accepted for the inactivation of envelope RNA viruses, recent studies have demonstrated that prolonged heat inactivation is required to completely inactivate two alphaviruses, Western equine encephalitis virus and CHIKV. Incomplete inactivation of viruses poses a laboratory biosafety risk and can also lead to spurious test results. Despite its importance in ensuring the safety of laboratory personnel as well as test integrity, systematic investigation on the thermostability of alphaviruses has not been performed. In this study, the temperature tolerance and heat inactivation profiles of RRV, Barmah Forest, and o’nyong-nyong viruses were determined. Variations in thermostability were observed within the Semliki forest serocomplex. Therefore, evidence-based heat inactivation procedures for alphaviruses are recommended.

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1. Introduction

Alphaviruses are enveloped, single-stranded, positive-sensed RNA viruses in the family Togaviridae. The public health significance of alphaviruses has been well recognized as several alphaviruses have become emerging and re-emerging pathogens in both the Old and New World. Infections with Old World alphaviruses often lead to arthritis whereas New World alphavirus infections generally cause encephalitic diseases. The disease burden caused by Old World alphaviruses has substantially increased. The most notable example is chikungunya virus (CHIKV). Since the 2004 outbreak on the islands of the Indian Ocean, CHIKV has spread to the Caribbean and the Americas in 2013 causing millions of cases along with ongoing re-emerging infections in other affected regions, such as Southeast Asia and India (Higgs and Vanlandingham, 2015). In addition to CHIKV, other alphaviruses such as Ross River virus (RRV), Barmah Forest virus (BFV) and o’nyong-nyong virus (ONNV) are medically important re-emerging alphaviruses associated with arthralgia syndrome. The two Australian alphaviruses, RRV and BFV, have historically had an estimated incidence rate of approximately 5000 and 1000 cases per year, respectively (Russell, 2002; Suhrbier et al., 2012). However, there has been a significant increase in recent caseloads reported for both viruses. Between January and April of 2015, more than 6400 RRV cases were reported (Herriman, 2015). Similarly, BFV reached a new average of 1700 reported cases per year between 2007 and 2012, peaking at 2080 in 2008 (Australian Government Department of Health, 2012). Although no major ONNV outbreaks have been reported since the epidemic in Uganda in 1996–1997, asymptomatic infections have led to silent transmission in several countries in Africa (Fokam et al., 2010; LaBeaud et al., 2015; Posey et al., 2005; Suhrbier et al., 2012; Tigoi et al., 2015).

Identification of asymptomatic infections by serological assays is critical for the understanding of the inter-epidemic viral maintenance and disease surveillance of alphaviruses prior to epidemics. This is also true for other emerging pathogens, for example the flavivirus Zika, since asymptomatic cases may develop viremias that may lead to blood transfusion infections (Kashima et al., 2016).
Among all the available serological assays, plaque reduction neutralizing test (PRNT) has been considered the gold standard by directly demonstrating the neutralizing capability of serum samples. Samples tested by PRNT must be inactivated at 56 °C for at least 30 min prior to experimental procedures, as recommended by the World Health Organization (WHO), to inactivate serum complement activities and contaminating viruses (Roehrig et al., 2008). Although effective against flaviviruses, recent publications suggest this protocol may be insufficient for the complete inactivation of Western equine encephalitis virus and CHIKV (Fang et al., 2009; Huang et al., 2015). Higher thermo-tolerance of alphaviruses may lead to false negative results when appropriate serum-only controls are not included. Additionally, the presence of residual infectious viruses in heat treated samples can be hazardous to laboratory personnel. It is important to determine the thermostability of these viruses in order to minimize interference with the neutralization test integrity and better inform biosafety procedures.

In this study, RRV, BFV, and ONNV were incubated at 56 °C to characterize their thermostability. Our results showed that alphaviruses have varying degree of thermostability and indicates the current standard heat inactivation protocol for alphaviruses should be virus-specific.

2. Materials and methods

2.1. Cell lines and virus

Stocks of RRV (T-48 strain) and BFV (AUS BH2 2193 strain) were propagated and harvested from C6/36 cell culture maintained in Leibovitz (L-15) medium as described previously (Huang et al., 2015). ONNV was derived from an infectious clone of the SG650 strain, followed by one passage in C6/36 cells (Vanlandingham et al., 2006). Titers of heat treated samples were determined using Vero cells maintained in L-15 medium using the standard procedures described by Higgs et al. (Higgs et al., 2006).

2.2. Heat inactivation

Virus stocks were serially diluted ten-fold to mimic viremia range of 4–7 logTCID50/ml in 3 ml samples in 15 ml conical tubes and heat treated in a pre-warmed water bath at 56 °C for 0, 20, 40, and 60 min. The heating period was extended to 140 min at 20 min intervals for viruses that survived the 60 min heat treatment to investigate their minimum time required for complete inactivation. At least four independent sets of samples were produced to test the reproducibility of the results.

2.3. Statistical analysis

To account for the associations resulting from repeated measurements from the same tube, linear mixed effects models were used to examine the effect of heating period on the thermostability of alphaviruses, controlling for the initial viral titers and the different alphaviruses. A multiple comparison test coupled with Tukey-Kramer adjustment was also performed as a post-hoc for further investigations on the differences among the alphaviruses and various heating periods. All data analyses were conducted using SAS software (version 9.4, Cary, NC) and data graphical display was carried out using R software (version 3.0.1). Results were considered statistically significant only if p < 0.05.
3. Results

3.1. Ross River virus

The heat inactivation profile of RRV at 56 °C was examined with initial titers of 6.95, 6.24, 5.23, and 4.24 logTCID₅₀/ml, as summarized in Fig. 1A. At the average input titer of 6.95 logTCID₅₀/ml, mean residual viral titers of 3.26, 2.37, and 1.48 logTCID₅₀/ml were observed at 20, 40, and 60 min after heat treatment, respectively. Mean initial viral titer of 6.24 logTCID₅₀/ml decreased to average titers of 1.95, 1.13, and 0.58 logTCID₅₀/ml after 20, 40, 60 min of heat inactivation, respectively. The heat inactivation of 5.23 logTCID₅₀/ml resulted in 1.40 logTCID₅₀/ml at 20 min, 0.42 logTCID₅₀/ml at 40 min, and 0.20 logTCID₅₀/ml at 60 min. With the average initial titer of 4.24 logTCID₅₀/ml, viral titers at 1.06, 0.51, and 0.35 logTCID₅₀/ml were observed at 20, 40, and 60 min after heat treatment, respectively. The presence of infectious virions at 40 min after heat treatment at 56 °C (t = 6.47, p < 0.001) indicated the standard heat inactivation protocol is insufficient to completely eliminate RRV from serum samples.

3.2. Barmah Forest virus

The temperature tolerance of BFV was determined using the following average input viral titers: 7.24, 6.24, 5.24, and 4.38 logTCID₅₀/ml, as summarized in Fig. 1B. Heat treatment at 56 °C of 7.24 and 6.24 logTCID₅₀/ml produced mean viral titers of 0.27 and 0.46 logTCID₅₀/ml, respectively, at 20 min. No infectious virions were recovered subsequently after 40 and 60 min of heat treatment. No residual viral titers were present in samples with average initial viral titers of 5.24 and 4.38 logTCID₅₀/ml at 20, 40, and 60 min after the initiation of heat inactivation (p = 0.292, 0.497, and 0.497, respectively). Therefore, BFV was successfully inactivated by the heat treatment procedure set by WHO (Roehrig et al., 2008).

3.3. O’nyong-nyong virus

Average initial titers of ONNV were 7.46, 6.33, 5.33, and 4.38 logTCID₅₀/ml. Results are shown in Fig. 1C. At a mean titer of 7.46 logTCID₅₀/ml, average residual titers of 2.12, 0.65, and 0.27 logTCID₅₀/ml were recovered at 20, 40, and 60 min after heat treatment, respectively. Stocks at an average initial titer of 6.33 logTCID₅₀/ml generated mean viral titers of 1.00 logTCID₅₀/ml at 20 min, 0.49 logTCID₅₀/ml at 40 min, and 0 logTCID₅₀/ml at 60 min of heat treatment. At the average viral titer of 5.33 logTCID₅₀/ml, residual titers declined to 1.57, 0.21, and 0 logTCID₅₀/ml after incubation for 20, 40, and 60 min, respectively. Mean viral titer of 4.38 logTCID₅₀/ml was recovered after heat treatment of initial viral titer of 1.01 logTCID₅₀/ml for 20 min. Infectious ONNV particles were not present after 40 and 60 min at 56 °C (p = 0.049 and 0.688, respectively), indicating that heat inactivation at 56 °C for 40 min is sufficient for ONNV.

3.4. Thermo-tolerance comparison of RRV, BFV, and ONNV

Heat inactivation of RRV, BFV, and ONNV demonstrated that the complete heat inactivation of RRV at 56 °C cannot be achieved by the currently implemented heat inactivation protocol. In contrast to RRV, BFV and ONNV are sensitive to heat inactivation. Based on multiple comparison tests with Turkey-Kramer adjustment, there was no difference between ONNV and RRV at 20 min (p = 0.836), but both were significantly more thermostable than BFV (both p < 0.001). Ultimately, BFV was significantly more heat sensitive compared to ONNV and RRV at 20 min. At 40 min, RRV was still significantly thermostable compared to ONNV and BFV (p = 0.0413 and p < 0.001, respectively). There was no difference between ONNV and BFV at 40 min (p = 0.704), since both their residual titers declined to zero.

3.5. Complete heat inactivation of Ross River virus

To determine the minimum time to achieve complete inactivation of RRV, another four sets of samples with average input titers of 7.27, 6.31, 5.24, and 4.29 logTCID₅₀/ml were heat treated at 56 °C for up to 140 min at 20 min intervals. Results are summarized in Fig. 2. In the group receiving average inoculum of 7.27 logTCID₅₀/ml, heat treatment led to 2.14, 1.89, 0.71, 1.18, 0.81, 0.33, and 0 logTCID₅₀/ml at 20, 40, 60, 80, 100, 120, and 140 min, respectively. Heat treatment of mean input titer of 6.31 logTCID₅₀/ml for 20, 40, 60, 80, 100, 120, and 140 min resulted in average viral titers of 1.61, 1.02, 0.38, 0.91, 0.27, 0, and 0 logTCID₅₀/ml, respectively. Residual viral titers of 0.90 and 0.30 logTCID₅₀/ml were recovered at 20 min after heat treatment at 56 °C with the average initial viral titers of 5.24 and 4.29 logTCID₅₀/ml, respectively. No infectious viri-
ons were detected after 40 and 60 min of heat inactivation at 56 °C for both groups starting with 5.24 and 4.29 logTCID50/ml.

4. Discussion

This study demonstrates the three alphavirus types tested show different thermal inactivation profiles. Virus-specific temperature tolerance was observed in the heat treatment of RRV, BFV, and ONNV. As suggested by previous studies, the current standard heat treatment procedure of 56 °C for 30 min was insufficient for the inactivation of all alphaviruses (Fang et al., 2009; Huang et al., 2015). Similar to CHIKV, greater than 120 min of incubation at 56 °C is required to inactivate RRV (Huang et al., 2015). Although ONNV is closely genetically and antigenically related to CHIKV, ONNV was completely inactivated by 60 min at 56 °C, demonstrating intra-clade variation in thermostability within the Semiliki Forest serocomplex (Powers et al., 2001; Powers et al., 2000; Suhrbier et al., 2012). Furthermore, BFV, which forms its own serocomplex (Powers et al., 2001), demonstrated even lower temperature tolerance, than ONNV, becoming undetectable by 20 min at 56 °C.

Our results highlight the importance of including serum-only controls when the standard heat inactivation protocol at 56 °C for 30 min is implemented, especially for RRV and ONNV. Whilst the increase in temperature could potentially achieve faster inactivation of thermostolerant alphaviruses, heat inactivation at greater than 56 °C is generally discouraged due to denaturation and gelatination of samples that may occur and interfere with neutralization tests (Vermeer and Norde, 2000). Under these circumstances, modification of the incubation time for heat inactivation and implementation of serum-only controls can ensure the sensitivity of neutralization tests based on the stability of immunoglobulin at 56 °C. Our findings have demonstrated that the temperature tolerance of several medically important Old World alphaviruses can vary. Further experimentation to determine the temperature tolerance of several New World alphaviruses is a significant gap in knowledge which should be filled.

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