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Development of a SYBR green I-based quantitative RT-PCR for Ross River virus: Application in vector competence studies and antiviral drug evaluation

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A B S T R A C T

Ross River virus (RRV) is an emerging *Alphavirus* and is presently endemic in many parts of Oceania. Keeping in mind its emergence, we developed a molecular detection system and utilized it to study vector competence and evaluate activity of antiviral compounds against RRV. A SYBR Green I-based quantitative RT-PCR for detection of RRV was developed targeting the E2 gene, with a detection limit of 100 RNA copies/reaction. The specificity was confirmed with closely related Alphaviruses and Flaviviruses. The assay was applied to study the vector competence of Indian *Aedes aegypti* for RRV, which revealed 100% infection and dissemination rate with 75% transmission rate. Viral RNA was found in saliva as early as 3 day post infection (dpi). Further application of the assay in antiviral drug evaluation revealed the superior *in vitro* activity of ribavirin compared to chloroquine in Vero cells. Successful demonstration of this assay to detect RRV in low titre mosquito samples makes it a sensitive tool in vector surveillance. This study also showed that Indian *Ae. aegypti* are well competent to transmit RRV highlighting the risk of its introduction to naïve territories across continents. Further validation of this assay, revealed its utility in screening of potential antivirals against RRV.

1. Introduction

Ross River virus (RRV) is an arthropod transmitted virus that is maintained through endemic and enzootic circulation in Australia, Papua New Guinea and Western Pacific Islands (Russell, 1994). The virus was first isolated in 1959 from *Aedes vigilax* mosquitoes near Ross River in Townsville, Queensland, Australia (Doherty et al., 1963). It has caused vast epidemics in 1979 and 1980 in Pacific islands *viz.* Fiji, New Caledonia, Samoa and Cook Islands (Kay and Aaskov, 1989). Detection of RRV specific IgG antibodies from blood donors supports the existence of autochthonous RRV transmission and silent circulation in French Polynesia (Aubry et al., 2015).

RRV belongs to genus *Alphavirus,* family *Togaviridae*. Its genome is single-stranded positive sense RNA that is 11.8 kb in length. It codes for two ORFs. The first ORF codes for four nonstructural proteins (nsP1, nsP2, nsP3 and nsP4). The second ORF codes for structural proteins including capsid and three envelope glycoproteins (E1, E2 and E3). Capsid protein and the genome together form the nucleocapsid. E1 and E2 viral glycoproteins are embedded in the lipid bilayer to form the envelope. The E3 glycoprotein is not incorporated in the virion and is released as a cleavage product (Strauss and Strauss, 1994).

RRV is an old world *Alphavirus* that has been grouped with Selmiki Forest virus (SFV), Chikungunya virus (CHIKV), O'nyong–nyong virus (ONNV), Barmah Forest virus (BFV), Ndumu virus (NDUV) (Solignat et al., 2009). These old world Alphaviruses are primarily arthritogenic in nature. RRV causes a self-limiting disease, which is characterized by acute and chronic polyarthralgia, polyarthritis, with acute manifestations including fever, myalgia, rash etc. Other symptoms include fatigue, headache, photophobia, lymphadenopathy, sore throat and rarely encephalitis (Suhrbier et al., 2012).

There are different *Aedes* species including *Ae. vigilax*, *Ae. alboannulatus*, *Ae. polynesiensis* incriminated to be the major vectors for RRV (Gard et al., 1973; Russell et al., 1991; Lindsay et al., 1997; Rosen et al., 1981). Apart from field isolations, experimental infection of different species of *Aedes* of varying geographical locations

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with RRV was performed. Experimental infection of *Ae. aegypti* from Queensland (Kay et al., 1979), Jakarta (Gubler, 1981) demonstrated their transmission potential for RRV. Subsequent study from Fiji in *Ae. aegypti* resulted in transmission rates ranging from 52 to 85% (Mitchell and Gubler, 1987). These early experiments demonstrated susceptibility and transmissibility of RRV in different *Aedes* species.

The detection of RRV from field collected mosquitoes can be achieved through various assays like virus isolation, immunological and molecular tests. Viral isolation is the gold standard technique, however it is time consuming, requires cell culture facility and technical expertise. Serological test can be done through antigen capture ELISA but suffers from issue of sensitivity to detect low viral titre associated with mosquito samples. In contrast, PCR based molecular assay provide faster results, sensitive in detecting low titre samples and also do not require containment facility. The real time quantitative RT-PCR is more advantageous, as quantitation can be simultaneously achieved. Further these techniques provide higher sensitivity and simplicity in terms of sample processing compared to conventional RT-PCR. This technique is now widely used for mosquito surveillance and to study virus vector interactions (Dash et al., 2012; Agarwal et al., 2013). Compared to another real time PCR assay based on Taqman probe, SYBR Green chemistry provide advantages in terms of cost, ease of performance and applicability for highly mutating RNA viruses. Low cost of this assay due to use of only desalted primers (expensive probes are not required) makes it economical for testing large number of samples. More importantly, this assay is insensitive to nucleotide variations that could occur within the probe target region, resulting in lower false-negative results (Papin et al., 2004).

In view of its current prevalence, there is an urgent need to develop specific antivirals to manage the patients. Currently, no licensed antiviral drug or vaccine for RRV is available. Only symptomatic treatment including anti-pyretic, anti-inflammatory and corticosteroids are prescribed to patients to ameliorate the disease (Mylonas et al., 2004). Development of antiviral remains one of the most important challenging areas in the management of viral infections. Classically, evaluation of antiviral compounds is performed employing plaque reduction assay. However, this technique is time consuming, labour intensive, making it restricted to advanced laboratories (Wei et al., 2013). Subsequently, other assays including immunofluorescence and antigen ELISA have also been utilized for antiviral screening. However, these techniques are not very sensitive, labour intensive and also requires specific antibody. The higher level of sensitivity and specificity achieved in PCR based molecular assays provides a convenient alternative for rapid screening of antivirals.

Ribavirin and Chloroquine are widely used antivirals for several families of viruses. Antiviral effect of chloroquine has been reported against several viruses including Chikungunya virus, Hepatitis C virus, Crimean-Congo Haemorrhagic fever virus, Human Immunodeficiency virus, Selmiki forest virus, SARS coronavirus (Khan et al., 2010; Farias et al., 2015; Helenius et al., 1982). Similarly, Ribavirin has been shown to inhibit Dengue virus, Chikungunya virus, Orthopoxviruses, Hantaan virus, Bornavirus, Canine Distemper virus etc (Smee et al., 2001; Takhampunya et al., 2006). Keeping in view their effectiveness against other members of *Alphavirus*, these two compounds are investigated for their antiviral activity against RRV.

International travel of RRV infected human from endemic area to a newer area can lead to establishment of RRV infection through transmission among local competent vectors. Importation of tires containing infected mosquito eggs from infested countries has led to virus establishment in naïve area (Benedict et al., 2007). Because of the naive population and lower herd immunity, virus can rapidly spread and cause major epidemics. Also, the diagnostic capabilities for Ross River virus in many non-endemic countries are lacking. Keeping these events in view, we developed a detection system for RRV in this study and utilized it for vector competence studies and antiviral drug evaluation.

2. Materials and methods

2.1. Ross river virus and mosquitoes

Ross River virus T48 strain obtained from Prof. Kouichi Morita, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan (GenBank Acc. No. GQ433359) was passaged thrice in C6/36 cells at Virology Division, Defence Research & Development Establishment (DRDE), Gwalior, India. Viral titre was found to be log 8 PFU/mL through plaque assay (Flint et al., 2004) in Vero cells (African green monkey kidney cells). The virus was aliquoted and stored in −80 °C until use. For cross-reactivity studies, among the alphaviruses, Chikungunya virus, DRDE-06 (EF210157) was used, while among the closely related flaviviruses viz., West Nile virus, Eg101(AF260968), Dengue virus-4, ND73(HM237348), and Japanese encephalitis virus, JaOArS982(M18370) were used. All these viruses were propagated in C6/36 cells in Virology Division, DRDE, Gwalior. Further, *in vitro* transcribed RNA derived from synthetic gene constructs of O'nyong-nyong virus (Gulu strain, M20303.1), Semliki Forest virus (L10 strain, AY112987) and Sindbis virus (J02363.1) were also used for cross-reactivity study due to non-availability of other alphaviruses.

Ae. aegypti used in this study were collected from Gwalior district, India in 2010 and maintained in Vector Management Division, DRDE at 28 ± 2 °C with 80% relative humidity and 14:10 light:dark photo period. 10% sucrose solution soaked in cotton pads was provided to adult mosquitoes.

2.2. SYBR green I-based real time RT-PCR

The primers for the SYBR Green I-based Real time RT-PCR assay were designed based on the identification of conserved sites through multiple sequence alignment of complete nucleotide sequence of ten available RRV isolates. Final modified primer pair consisted of forward primer: RRV8956S: 5′-TACAAGCACGACCCATTGCCG-3′ and reverse primer: RRV9162AS: 5′- GATAGTCCTGCCGCCTGCTGT −3′ targeting to a highly conserved region of E2 gene (Sellner et al., 1994). One-step quantitative RT-PCR was carried out using SS III Platinum one step qRT-PCR kit (Invitrogen, USA) in Mx3005 P system (Stratagene, USA). Briefly, reaction was carried out in a 25 μL volume containing 2X Master mix (12.5 μL), 0.25 μM (final concentration) each of forward and reverse primers (RRV8956S and RRV9162AS) (0.125 μL), enzyme mix comprising of Taq DNA polymerase and reverse transcriptase (0.25 μL), nuclease free water (9.5 μL) and RNA (2.5 μL). The thermal profile consisted of 30 min of reverse transcription at 50 °C, 10 min of polymerase activation at 95 °C, followed by 40 cycles of PCR at 95 °C for 30 s, 56 °C for 60 s, and 72 °C for 30 s. After amplification, a melting curve analysis was performed with the melting curve analysis software of Mx3005 P according to the manufacturer's instructions. Positive and negative template controls were also included along side in all experiments. Analysis of cycle threshold (Ct) values was performed for different samples.

2.3. Construction of standard curve and quantification of RNA transcripts

PCR amplicon of RRV E2 gene was generated using a modified E2 forward primer (T7 promoter sequence-TAATACGACTCACTATAGG was added at the 5′ end of RRV8956S) and normal RRV9162AS. The amplicon of 226 bp was gel purified, quantitated using nanodrop