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.

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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

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

spectrophotometer (Thermo Scientific, USA) before being used as template in transcription reaction. The purified template was subjected to *in vitro* transcription (IVT) using TranscriptAid T7High Yield Transcription Kit (Thermo Scientific, USA) at 37 °C for 1 h. The IVT products were then treated with 1 U of DNase I to remove the remaining DNA followed by inactivation of DNase I. The IVT products were ethanol precipitated and resuspended in DEPC treated water. The amount of RNA transcripts were determined spectrophotometrically and converted to molecular copies using the following formula.

$$Y(molecules/\mu L) = [X(g/\mu L)/transcript length(nucleotides)$$
$$\times 340] \times 6.023 \times 10^{23}$$

A standard curve was prepared by plotting cycle threshold (Ct) values vs. serial dilution of known concentrations of RNA transcripts ranging from 10^8 to 10^2 copies.

2.4. Conventional end-point RT-PCR

In order to compare the sensitivity of conventional RT-PCR and SYBR Green I-based Real time RT-PCR assay, a conventional RT-PCR was performed with the same primer sets targeting the E2 gene (RRV8956S and RRV9162AS). One-step conventional end-point RT-PCR was standardized using Enhanced Avian HS RT-PCR kit (Sigma, USA). The amplification was carried out in a 25 μL reaction volume comprising of 10X reaction buffer (2.5 μL), 25 mM MgCl₂ (1.5 μL), 10 mM dNTPs (0.5 μL), 0.5 μM (final concentration) each of forward and reverse primer (0.25 μL), Jump start AccuTaq DNA polymerase (0.25 μL), Enhanced avian reverse transcriptase (0.25 μL), Ribonuclease inhibitor (0.25 μL), nuclease free water (16.75 μL) and Template (2.5 μL). The thermal profile of RT-PCR reaction consisted of: cDNA synthesis–48 °C (45 min) initial denaturation–95 °C (5 min), followed by 40 cycles of denaturation–95 °C (1 min), annealing–56 °C (1 min), extension–72 °C (1 min) and final extension–72 °C (10 min) in a thermal cycler (Applied Biosystems, USA). PCR products were then electrophoresed on 2% agarose gel and visualized on a Gel documentation system (BioRad, USA).

2.5. Oral infection of mosquitoes

Female *Ae. aegypti* (4–5 days old) were used for infection purpose. They were starved for 24 h prior to infectious blood meal so as to enhance blood feeding. The infectious blood meal was prepared by adding 1 mL of RRV culture supernatant in 2 mL of washed rabbit erythrocytes. The final concentration of RRV in blood meal was 3.3×10^7 PFU/mL. ATP was added as a phagostimulant to final concentration of 5 mM. Mosquitoes were orally infected through membrane feeding using warm circulating water as described previously (Agarwal et al., 2013). After feeding, five fully engorged *Ae. aegypti* were collected for titration of imbibed virus. Eight surviving *Ae. aegypti* were processed on 3, 6, 8, 10, 12, 14 dpi to determine infection, dissemination and transmission rates as described previously (Agarwal et al., 2013).

2.6. Mosquito processing and viral RNA extraction

Mosquito organs (midgut, legs & wings) from orally infected individual *Ae. aegypti* mosquito were triturated in 2 mL tubes with 300 μL of Eagles Minimum Essential Medium (EMEM) (Sigma, USA) and stainless steel beads using TissueLyser LT (Qiagen, Germany). The homogenate was clarified by centrifugation at 6000 x *g* for 10 min and 140 μL of supernatant was used to extract RNA using QIAamp viral RNA mini kit (Qiagen, Germany) according to the

manufacturer's protocol. Saliva from infected mosquitoes was expectorated using an artificial salivation technique for evaluating transmission potential (Agarwal et al., 2013). 140 μL of media containing saliva from individual mosquito was processed similarly. The RNA was eluted in 50 μL elution buffer. RNA from mosquito samples was subjected to RRV specific SYBR Green I-based real time RT-PCR as described earlier.

2.7. Detection of RRV in mosquito pool

During vector surveillance, mosquitoes are generally analyzed in pools. Therefore, a sensitive detection system is required that can detect even presence of a single infected mosquito in different pool sizes. The applicability of SYBR Green I-based real time RT-PCR for screening of mosquito pools was investigated by adding one infected *Ae. aegypti* from 8 dpi to different pool sizes of uninfected mosquitoes (9, 19, 29, 39, 49, 59, 79 and 99). The pooled mosquitoes were homogenized as mentioned earlier and the supernatant was used for the extraction of RNA.

2.8. Cell viability (Neutral red dye uptake) assay

To determine maximum non-toxic dose (MNTD) of Chloroquine and Ribavirin, cell viability assay was carried out. Briefly, 96 well plates (Eppendorf, Germany) were seeded with Vero cells and incubated at 37 °C with 5% CO₂. After 24 h, different concentrations of Chloroquine (200 μM, 100 μM, 50 μM, 25 μM, 12.5 μM, 6.25 μM, 3.125 μM, 1.562 μM) and Ribavirin (2000 μM, 1000 μM, 500 μM, 250 μM, 125 μM, 62.5 μM, 31.25 μM, 12.62 μM) were added in cells and observed for 48 h. After 48 h, percent cell viability was determined by Neutral Red Dye Uptake assay (NRDU) (Finter, 1969).
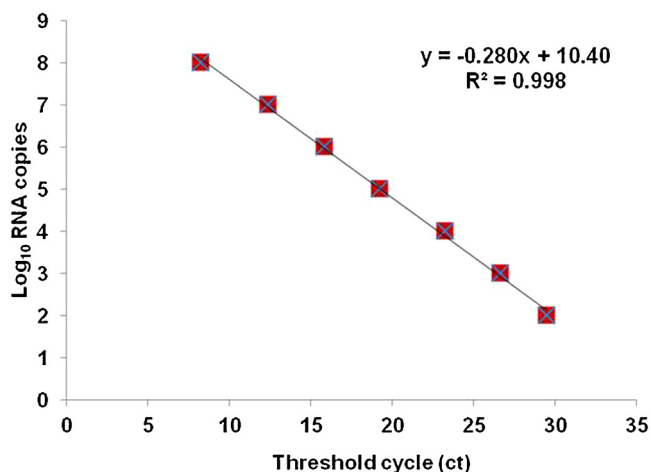
2.9. Ross river virus inhibition assay

Vero cells were plated in 24 well plate (Eppendorf, Germany) and confluent monolayer cells were infected with 0.01 moi of RRV. Cell controls were also kept alongside. After 2 h adsorption, infected medium was removed, cells were washed with PBS and replenished with EMEM. Chloroquine and Ribavirin were added at their respective maximum non-toxic dose. Cells were observed up to 48 h post infection (hpi) for any detectable morphological changes. Supernatants from virus infected and drug treated cells were harvested at 24 and 48 hpi.

2.10. Quantification of viral genomic RNA by quantitative real time RT-PCR

RNA was extracted from 140 μL of cell supernatant using QIAamp viral RNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. RNA was eluted in 50 μL of nuclease free water and then subjected to SYBR Green I-based real time RT-PCR using SS III Platinum one step qRT-PCR kit (Invitrogen, USA) in Mx3005 P system (Stratagene, USA) as described earlier.

2.11. Statistics

Data is represented as Mean ± standard deviation. For virus-mosquito experiments, values are mean of eight replicates at one time point. For virus inhibition assays, values are mean of three replicates from three independent experiments. The difference between the groups was statistically analyzed by Student's *t*-test (two-tailed, unpaired) and One-way ANOVA with post-hoc Tukey HSD Test. A *p*-value of less than 0.05 was considered significant. $p < 0.05$ is represented by *, $p < 0.01$ is represented by **, $p < 0.001$ is represented by ***.

Fig 1. Standard curve drawn from 10 fold serial dilutions of known concentrations of RRV RNA. A linear range of 7 logs of dilutions with the coefficient of determination ($R^2 = 0.998$) and slope (m = −0.280) of the curve are indicated.

3. Results

3.1. Optimization of SYBR green I-based real time RT-PCR

The SYBR Green I-based real time RT-PCR assay was optimized using a set of primers targeting the highly conserved region of E2 gene of RRV. There is a very high degree of conservation in primer sequence of the target. (Fig. S1). Optimal annealing temperature of the assay was set at 56 °C with a final primer concentration of 0.25 μM. Melting curve analysis revealed a specific melting temperature (Tm) of 87.5 ± 0.2 °C for RRV specific amplicons.

3.2. Standard curve

Standard curve of RRV specific SYBR Green I-based real time RT-PCR was constructed using serial 10 fold dilutions of known concentrations of *in vitro* transcribed RRV RNA. A dynamic linear range of 7 logs of dilutions with the coefficient of determination ($R^2 = 0.998$) and slope (m = −0.280) of the curve was indicated (Fig. 1).

3.3. Sensitivity and specificity of real time RT-PCR

The detection limit of real time RT-PCR assay was found to be 100 copies/25 μL reactions, while the detection limit of endpoint RT-PCR was found to be 1000 copies/25 μL reaction (Fig. 2). The specificity of the assay was confirmed using RNA/*in vitro* transcribed RNA from several closely related alphaviruses and flaviviruses. No amplification was observed from the viruses tested in this study (data not shown).

3.4. Detection and quantitation of RRV in mosquito pool

The detection efficiency of real time assay to identify one infected mosquito in different pool sizes was evaluated. It was observed that the assay was successful to detect even a single infected mosquito in a pool up to 79 uninfected mosquitoes. The mean titre of RRV RNA in different pools was found to be $10^{4.7} \pm 10^{0.2}$ RNA copies/reaction (Fig. 3).

3.5. Assessment of RRV specific SYBR green I-based real-time RT-PCR by studying replication kinetics in Ae. aegypti

3.5.1. Analysis of midgut for RRV infection

RRV infection rate was studied by titrating viral RNA in mosquito midgut, which was found to be 100% at all the analyzed time points. Viral RNA titre in midgut is measured in terms of $\log_{10}$ RNA copies/midgut. Initial mean titre of RRV engorged by mosquitoes was found to be $10^{6.0} \pm 10^{0.5}$. At 3 dpi, viral RNA titre was $10^{7.0} \pm 10^{1.5}$, which slightly decreased to $10^{5.9} \pm 10^{-1.9}$ at 6 dpi and then highest titre was observed at 8 and 10 dpi with $10^{7.6} \pm 10^{1.8}$ and $10^{7.6} \pm 10^{1.5}$ respectively. From 12 dpi onwards, viral RNA titre slightly declined and reached to $10^{7.1} \pm 10^{1.9}$ at 14 dpi (Fig. 4a).

3.5.2. Analysis of legs & wings for RRV dissemination

RRV dissemination rate was studied by titrating viral RNA in mosquito's legs and wings, which was found to be 100% at all the analyzed time points. Viral RNA titre in legs & wings is measured in terms of $\log_{10}$ RNA copies/legs & wings. At 3 dpi, viral RNA titre was found to be $10^{5.6} \pm 10^{1.6}$ that remained at $10^{5.1} \pm 10^{1.4}$ and $10^{6.3} \pm 10^{0.9}$ RNA copies at 6 and 8 dpi respectively. At 10 dpi, highest viral RNA load of $10^{6.5} \pm 10^{1.2}$ was achieved, which later decreased slightly to $10^{5.9} \pm 10^{1.2}$ and $10^{6.1} \pm 10^{1.2}$ at 12 and 14d pi respectively (Fig. 4b).

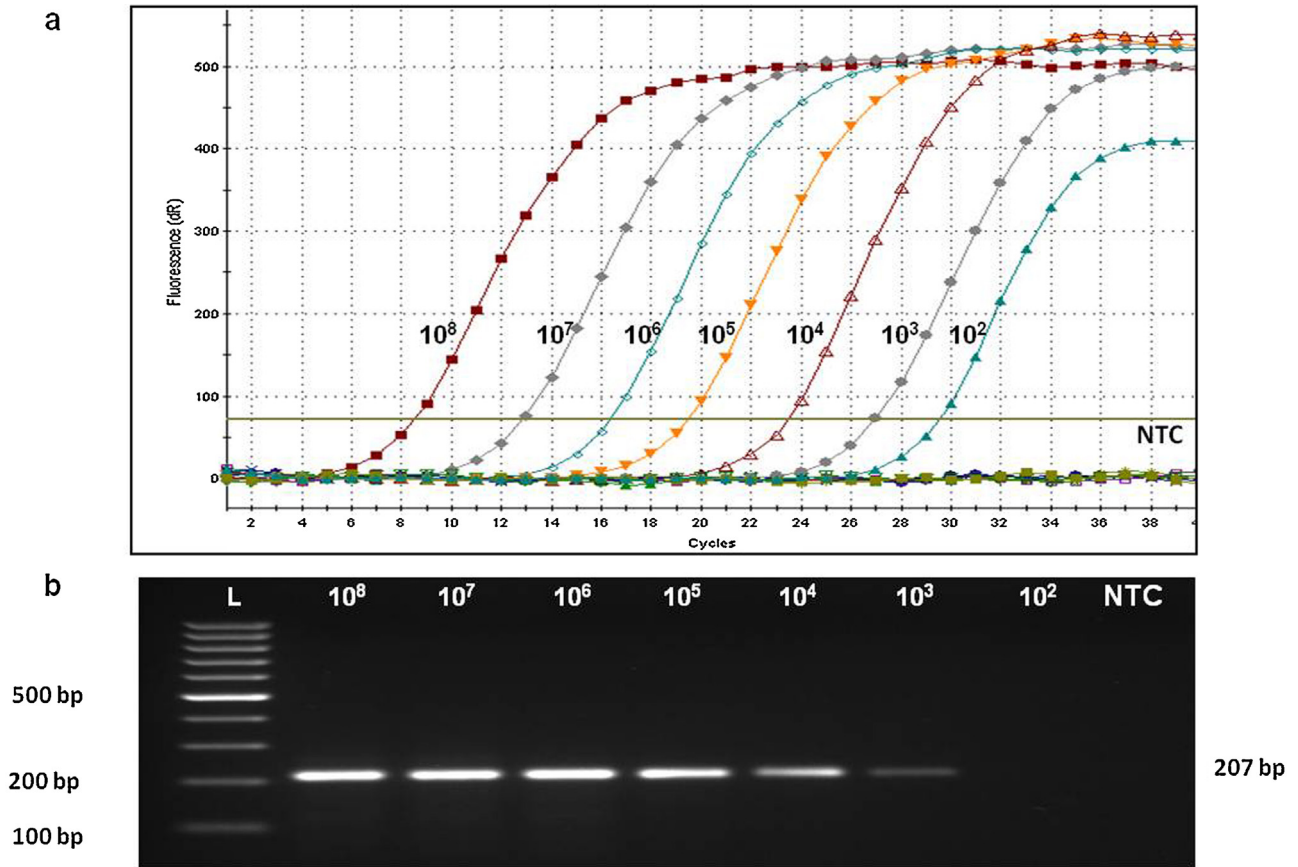#### 3.5.3. Analysis of saliva for RRV transmission

RRV transmission rate was studied by titrating viral RNA in mosquito saliva, which was found to be 87.5% at 14 dpi. Viral RNA titre in saliva is measured in terms of $\log_{10}$ RNA copies/saliva. RRV was detected in mosquito saliva from 3 dpi, however the titre was low i.e. $10^{1.6} \pm 10^{2.2}$, which showed slight increment at 6 and 8 dpi with $10^{1.7} \pm 10^{1.9}$ and $10^{1.7} \pm 10^{1.8}$ respectively. It reached its peak at 10 dpi, with $10^{3.3} \pm 10^{1.3}$. The titre remained $10^{3.3} \pm 10^{1.9}$ at 12 dpi which subsequently decreased to $10^{2.6} \pm 10^{1.8}$ at 14 dpi (Fig. 4c).

3.5.4. Assessment of RRV specific SYBR green I-based real-time RT-PCR by studying virus inhibition assays

Maximum non-toxic dose (MNTD) of chloroquine and ribavirin was found to be 25 μM and 125 μM respectively in Vero cells as cell viability was >85% in Chloroquine and ribavirin at these concentrations (Fig. 5a, b). The mean RNA titre in RRV infected cells was found to be $10^{6.3} \pm 10^{1.1}$ at 24 hpi, while in chloroquine and ribavirin treated cells, the mean RNA titre was found to be $10^{5.6} \pm 10^{0.9}$ ($p = 0.48$) and $10^{4.0} \pm 10^{0.7}$ ($p = 0.04$) respectively. At 48 hpi, the mean RNA titre in RRV infected cells was found to be $10^{7.1} \pm 10^{0.5}$, while in chloroquine and ribavirin treated cells was found to be $10^{6.9} \pm 10^{0.1}$ ($p = 0.72$) and $10^{5.9} \pm 10^{0.7}$ ($p = 0.11$) respectively (Fig. 5c). Percent reduction in RRV viral RNA expression in chloroquine and ribavirin treated cells at 24 hpi was found to be 66.1% ($p = 0.01$) and 99.2% ($p < 0.001$) respectively, compared to RRV infected cells. At 48 hpi, reduction in RRV viral RNA expression in chloroquine and ribavirin treated cells was found to be 37.9% ($p = 0.11$) and 91.5% ($p < 0.01$) respectively, compared to RRV infected cells (Fig. 5d).

4. Discussion

Ross River virus has been the most important arboviral threat in Australia since the first documented outbreak of RRV in 1979. Though major outbreaks has not been reported outside Australia, however there are imported cases of RRV infection in New Zealand, through travelers who had travelled to Australia or nearby islands (Lau et al., 2012). Recently cases of RRV infection were reported in Dutch and Japanese patient who returned from Australia (Tochitani et al., 2014; Reusken et al., 2015).
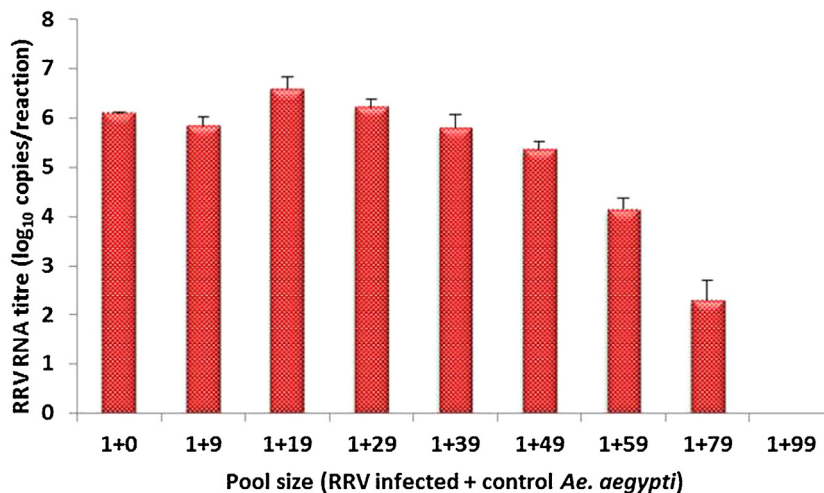
Fig. 2. Comparative sensitivity of RRV specific SYBR green I-based real time RT-PCR vs. end point RT-PCR: a. Amplification plots from left to right are the curves of 10-fold serial dilutions of known concentration of RRV RNA from 1×10^8 to 1×10^2 RNA copies/25 mL reaction. b. Sensitivity of end point RT-PCR for the detection of RRV RNA as demonstrated by 207 bp amplicon on agarose gel analysis. NTC— No template control, lane L- Ladder (100 bp). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
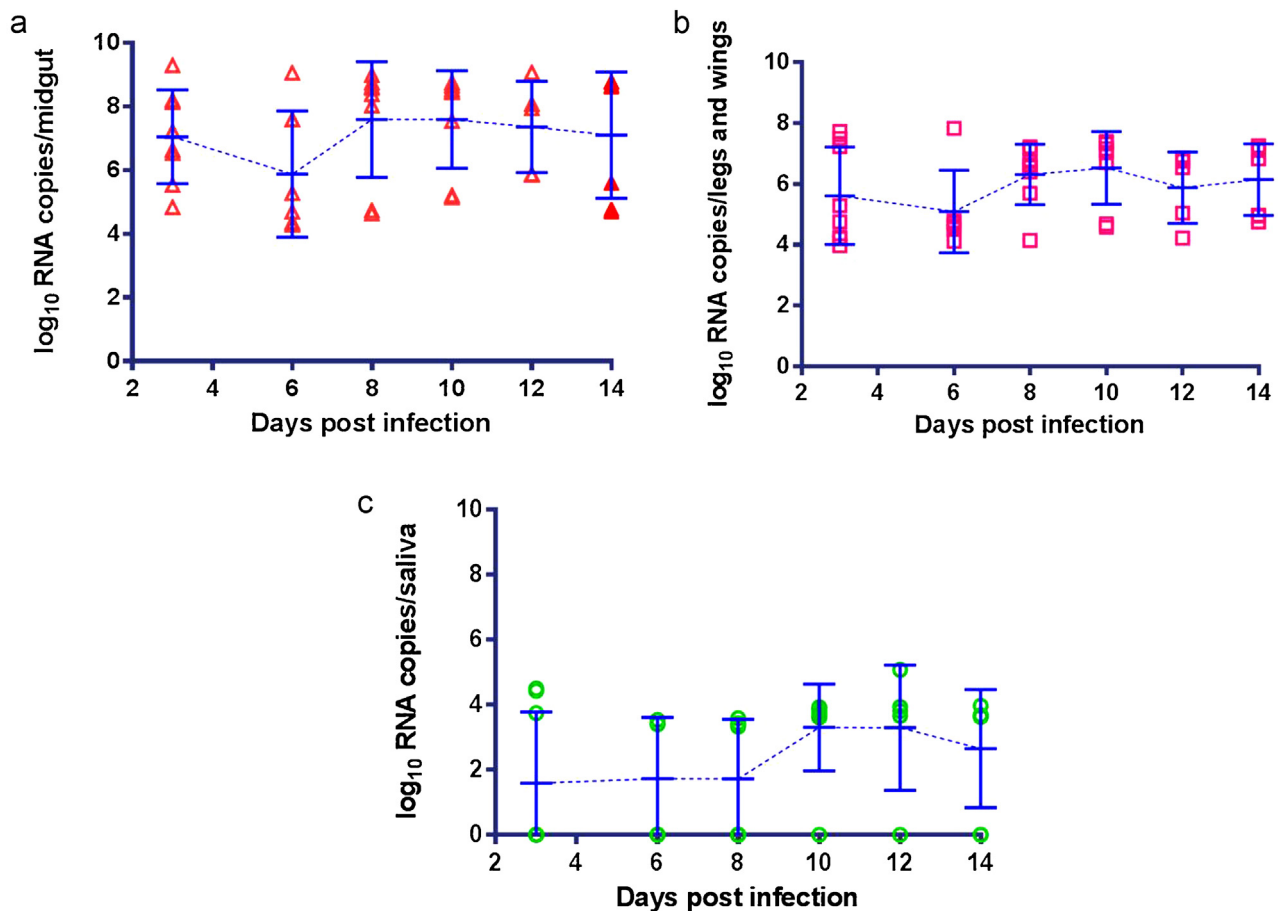
However, autochthonous transmission was not recorded in these cases unlike recent outbreaks of other arboviruses like Chikungunya and Zika virus (Grandadam et al., 2011; Maria et al., 2016). This might be due to the absence of vectors or presence of incompetent vectors in the imported territories. The current endemicity of RRV in Oceania and its potential risk for tropical countries, mandates the urgent development of a simple, sensitive and specific molecular detection technique.

In this study, our main objective was to develop a quantitative SYBR Green I-based real time RT PCR for detection of RRV and apply it in vector competence studies as well as in antiviral drug evaluation. Replication kinetics of RRV in Indian *Ae. aegypti* was measured by infection, dissemination and transmission at differ-

Fig. 3. Effect of increasing pool size of *Ae. aegypti* on detection and quantitation of RRV by RRV specific SYBR Green I-based real time real-time RT-PCR. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
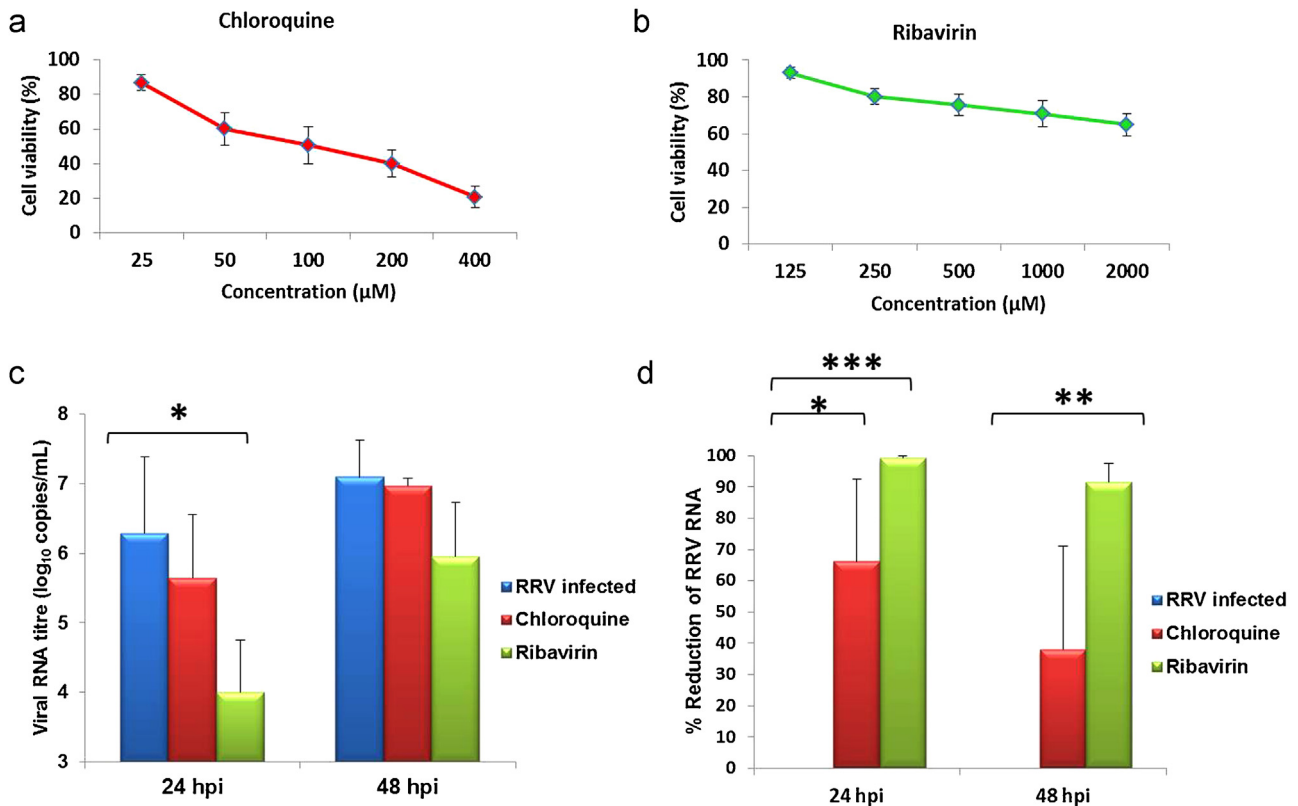
Fig. 4. a. RRV infection in *Ae. aegypti* b. RRV dissemination in *Ae. aegypti* c. RRV transmission by *Ae. aegypti*. Following oral infection of *Ae. aegypti* with blood meal containing RRV at a final concentration of 3.3×10^7 PFU/mL, infectivity was determined by measuring $\log_{10}$ RNA copies/midgut of each female by real time RT-PCR at different days of post infection. Dissemination was determined by measuring $\log_{10}$ RNA copies/legs. Transmission was determined by measuring $\log_{10}$ RNA copies/saliva. Replicates are represented by symbols. Replicates with connected mean are also indicated.

ent days of post infection. Infection rate was determined as the percentage of all mosquitoes tested with infected midgut, which was found to be 100% at all the analyzed time points. Dissemination rate was determined as the percentage of mosquitoes with infected midgut that also had infected legs and wings, which was also found 100% at all the analyzed time points. The transmission rate was determined as the percentage of mosquitoes with positive dissemination that also had infected saliva. The transmission rate was found to reach up to 87.5% on 10 dpi. At 14 dpi, 75% transmission rate was achieved, which was higher compared to CHIKV as reported previously (Agarwal et al., 2013). This high level of transmission rate clearly indicates very high vectorial competence of Indian *Ae. aegypti*. Transmission analysis revealed presence of RNA in mosquito saliva on 3 dpi indicating a short extrinsic incubation period. A short extrinsic incubation period is of very high significance in terms of faster virus transmission and may lead to major outbreak. Also, the assay was efficient to detect virus among mosquitoes of different pool sizes, demonstrating its utility during vector surveillance.

Studies evaluating vector competence/transmission ability of any Indian mosquito for RRV has not been conducted earlier. *Ae. aegypti* spread from Africa to tropical and subtropical regions across the globe, is the primary vector for many viruses including Chikungunya (CHIKV), Dengue (DENV), Yellow fever (YFV) and Zika virus (ZIKV) (Tabachnick, 1991). *Ae. aegypti* is the most dominant *Aedes* species widely distributed in all parts of India. The recent establishment of WNV, CHIKV and ZIKV in new territories substantiate the

real threat associated with arboviruses. These risks associated with vector borne diseases inspired us to develop a sensitive technique for detecting RRV in mosquitoes. Since specific antiviral drug or vaccine against RRV is not yet available, early detection in mosquitoes can be a potential indicator of circulation of virus in nature and will assist to initiate prompt control measures. This preliminary study provides information on the vector competence of Indian *Ae. aegypti* for RRV.

Our second objective was to assess the utility of developed qRT-PCR assay for studying *in vitro* antiviral efficacy of known antiviral compounds like chloroquine and ribavirin. Maximum non-toxic dose of chloroquine was found to be 25 μM in Vero cells. Previous studies on Chikungunya virus have demonstrated 5–20 μM as the effective concentration of chloroquine (Khan et al., 2010). However in this study, only marginal protection was observed with chloroquine against RRV. Different inhibitory mechanisms of chloroquine involves prevention of viral endocytosis (Khan et al., 2010), decrease in concentration of cytokines like TNF-α, IFN-γ that are associated with disease severity and decrease in systemic levels of a liver enzyme (Aspartate aminotransferase) (Farias et al., 2015). In contrast, ribavirin, a broad spectrum antiviral inhibits inosine monophosphate dehydrogenase (IMPDH), thus depleting intracellular GTP pool (Takhampunya et al., 2006). It is a broad spectrum antiviral agent that acts as a RNA virus mutagen, causing lethal mutations in RNA virus genome (Crotty et al., 2002). Earlier studies employing Vero cells have shown effective dose of 30–250 μM for orthopoxviruses (Smee et al., 2001), which is in agreement with

Fig. 5. Cell viability and Ross River virus inhibition assay. Maximum non-toxic dose (MNTD) of a. Chloroquine and b. Ribavirin were detemined by neutral red dye uptake (NRDU) cellular toxicity assay on Vero cells. c. RRV titre in RRV infected, chloroquine treated and ribavirin treated cells at 24 and 48 hpi. d. Percent reduction in RRV RNA expression in RRV infected, chloroquine treated and ribavirin treated cells at 24 and 48 hpi. $p < 0.05$ is represented by *, $p < 0.01$ is represented by **, $p < 0.001$ is represented by ***. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

this study. Ribavirin was found to provide effective protection up to 48 hpi. Further *in vivo* studies need to be carried out to evaluate the antiviral potential of Ribavirin against RRV.

5. Conclusions

The recent emergence and reemergence of different arboviral infections around the world makes the development of suitable sensitive diagnostics, one of the top most priority. The development of a highly sensitive real time RT-PCR based molecular assay has been shown to be extremely useful for rapid detection and quantitation of RRV. The assay was successfully applied for vector competence studies in mosquitoes as well as for evaluation of antiviral drugs against RRV. The results clearly revealed the very high vectorial competence of Indian *Aedes* mosquitoes indicating the threat of establishment of RRV infection in naïve *Aedes* endemic zone outside Australia. There is an urgent need to study vector competence of geographically diverse *Aedes* mosquitoes in order to understand their threat potential and to develop suitable control measures.

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