Construction of an infectious Chikungunya virus cDNA clone and stable insertion of mCherry reporter genes at two different sites

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Chikungunya virus (CHIKV) has caused massive epidemics in the Indian Ocean region since 2005. It belongs to the genus Alphavirus and possesses a positive-stranded RNA genome of nearly 12 kb in size. To produce genetically modified viruses for the study of various aspects of the CHIKV life cycle, a reverse genetic system is needed. We report the generation of a T7 RNA polymerase-driven infectious cDNA clone of CHIKV. Electroporation of in vitro-transcribed RNA resulted in the recovery of a recombinant virus with growth characteristics comparable to the parental strain. Using the established cDNA clone, the red fluorescent marker gene mCherry was introduced into two different sites within the CHIKV nsP3 gene. Both constructs allowed the rescue of stable fluorescent reporter viruses with growth characteristics similar to the wild-type virus. The latter reporter viruses represent valuable tools for easy follow-up of replicating CHIKV useful in several applications of CHIKV research.

Chikungunya virus (CHIKV) is a small arthropod-borne enveloped RNA virus in the genus Alphavirus, family Togaviridae (Kuhn, 2007). It is transmitted by culicids of the genus Aedes including Aedes aegypti and Aedes albopictus (Diallo et al., 1999). The genome is a single-stranded plus-stranded RNA with a size of about 11.7 kb, capped at the 5'-end and polyadenylated at the 3'-end (Kuhn, 2007). The 5’ two-thirds of the genome encodes the non-structural proteins (nsP1-nsP2-nsP3-nsP4). From the 3’ third, a subgenomic message is transcribed during replication encoding the structural proteins (C-E3-E2-6K-E1) (Kuhn, 2007).

CHIKV has caused large-scale epidemics in the Indian Ocean region starting from 2005. Studies of epidemic viruses indicated that mutations in the structural protein region played an important role in those epidemics (Sreekumar et al., 2010; Tsetsarkin et al., 2007). In particular, it was shown by reverse genetics that a single amino acid exchange in the structural E1 protein (A226V) could increase vector competence of the ubiquitous Ae. albopictus (Tsetsarkin et al., 2007). Recently, mutations in the receptor-binding E2 protein were also shown to be involved in vector specificity (Tsetsarkin et al., 2009). CHIKV reverse genetics can in addition be used to study replication, attenuation markers and immunogenicity, enabling the development of recombinant vaccines (Gorchakov et al., 2012; Plante et al., 2011; Wang et al., 2011). Furthermore, recombinant viruses encoding reporter genes represent useful tools to follow up viral replication and spread in cell culture, but also in animals or mosquitoes (Frolova et al., 2006; Pohjala et al., 2011; Tamberg et al., 2007; Ziegler et al., 2011).

For the construction of an infectious cDNA clone, CHIKV was isolated from a patient infected during an outbreak in Mauritius in 2006 and the complete sequence was determined (GenBank accession no. FJ959103) (Panning et al., 2008). Four long-range RT-PCR fragments covering the complete genome were amplified and cloned into the low copy vector pSMART-LCKan using the Clone SMART Blunt cloning kit (Lucigen) (Fig. 1). A T7 promoter sequence was added during the initial RT-PCR upstream of the 5’-end of the viral cDNA and a NotI linearization site was introduced downstream of the poly(A) tail at the 3’-end of the viral cDNA. The individual plasmids were fused stepwise using a restriction site within the vector sequence each time (Fig. 1). The resulting infectious full-length clone of the Mauritius isolate was named pCHIKV-M.

To test the functionality of pCHIKV-M, RNA was transcribed from the NotI linearized plasmid using the T7 mMESSAGE mMACHINE kit (Ambion). In vitro-transcribed RNA (1.5 µg) was electroporated into 1 × 10⁶ BHK cells suspended in 100 µl OptiPro-SFM medium (Gibco) in a 2 mm electroporation cuvette using the GenePulser X-cell (Bio-Rad) with one pulse of 140 V and a pulse length of 25 msec. Specific infectivity of 1.2 × 10⁵ p.f.u. µg⁻¹ of electroporated RNA was obtained, as determined by an infectious centre assay (Kümmerer & Rice, 2002). Electroporated
cells showed a specific immunofluorescence signal with human anti-CHIKV sera 24 h after electroporation and a clear cytopathic effect approximately 36 h post-electroporation (data not shown). Plaque titration of the supernatant at 24 h after electroporation on Vero cells using an agarose overlay yielded a titre of $3.4 \times 10^6$ p.f.u. ml$^{-1}$. The plaque size was comparable to that obtained for wild-type (wt) CHIKV isolated from the patient (Fig. 2a).

To analyse the growth characteristic of the rescued virus in further detail, the kinetics of replication in comparison to wtCHIKV was assessed by means of quantitative RT-PCR as described previously (Panning et al., 2008). Vero cells were infected with wtCHIKV or recombinant (r) CHIKV at an m.o.i. of 0.1 and the release of viral genome into the supernatant was compared. As can be seen in Fig. 2(b) wtCHIKV and rCHIKV showed comparable replication kinetics.

Fig. 1. Construction scheme of the CHIKV infectious cDNA clone. On top the genome of CHIKV is depicted, indicating the positions of restriction sites used for assembly of the full-length clone. White parts of the circles represent the sequence of the vector pSMART-LCKan, black parts represent genome segments of CHIKV. In addition, a T7 promoter was fused upstream of the 5’-end of the genomic sequence (grey box). The sizes of the subsegments are indicated for the first set of plasmids pA through pD. Stepwise cloning was performed via the restriction sites indicated in black letters. Restriction sites written in grey letters indicate the ligation positions. In all cases, cloning was performed via one restriction site in the viral sequence and one restriction site in the vector sequence. The plasmid containing the 5’ half of the genome (pCHIK-L) and the plasmid containing the 3’ half of the genome (pCHIK-R) were assembled to the final cDNA clone pCHIKV-M. The NotI site introduced at the 3’-end of viral genome is used for linearization before in vitro transcription.

Fig. 2. (a) Plaque morphology of wtCHIKV and rCHIKV. Plaque formation was analysed on Vero cells using an agarose overlay. Two days after infection, cells were fixed and crystal violet staining was performed. (b) Growth kinetics of wtCHIKV and rCHIKV. Vero cells were infected at an m.o.i. of 0.1. Quantification of viral RNA in the supernatant at the indicated time points was performed by real-time PCR. In vitro transcripts served as standard. (c) Verification of the genetic marker introduced into rCHIKV. Virus obtained after electroporation was passaged once in tissue culture. Infection with the field isolated CHIKV (wtCHIKV) was done for comparison. RT-PCR products derived from RNA of cells infected with the indicated viruses were either loaded directly (−) or after Scal restriction (+) on an ethidium bromide-stained agarose gel. Control, PCR without addition of reverse transcriptase.

To formally confirm the successful rescue of recombinant CHIKV, the genetic marker introduced into the infectious cDNA clone should be proven. To this end, fresh cells were
infected with the rescued rCHIKV and 24 h after infection, total cellular RNA was isolated using TRIzol (Invitrogen). For comparison, infection with the field isolate was performed. Isolated RNA was used as the starting material for RT-PCR using primers spanning the region containing a ScaI restriction site in the wt capsid gene (amplified fragment: nt 7476–8559 of the CHIKV genome). The latter restriction site had been deleted for the recombinant virus by silent mutagenesis (amino acid Y: TAC to TAT exchange). The amplified RT-PCR fragments were 1084 bp in size (Fig. 2c). These products could not result from carryover of plasmid DNA, since a control reaction without addition of reverse transcriptase did not yield a PCR product (Fig. 2c, control). Whereas the RT-PCR fragment could be digested for the field isolate by ScaI to yield two fragments of 677 and 407 bp in size, the corresponding fragment obtained after infection with rCHIKV remained undigested (Fig. 2c), proving the recombinant nature of the rescued virus.

Infectious cDNA clones represent a great starting point to establish reporter gene expressing viruses for easy follow-up. A common strategy used to establish reporter gene encoding alphaviruses is to express the gene of interest via a subgenomic promoter (Olson et al., 1994; Vanlandingham et al., 2005; Ziegler et al., 2011). However, such constructs tend to be unstable (Pugachev et al., 1995), probably because the inserted genes are transcribed from a separate subgenomic RNA, which in the case of reporter gene expression does not encode a product suitable for selection. Furthermore, homologous recombination between the subgenomic promoter can lead to deletion of the reporter (Pugachev et al., 2000).

Alternatively, reporter genes have been inserted directly into alphaviral genes. Besides insertion into the Sindbis virus (SINV) structural region (Thomas et al., 2003), several reports describe the introduction into the nsP3 gene of alphaviruses. Whereas insertion of EGFP after amino acid position 452 of Semliki Forest virus (SFV) nsP3 resulted in infectious virus, EGFP expressing SFV could be successfully passaged upon insertion of EGFP between nsP3 and nsP4 flanked by duplications of the nsP3/4 nsP2 protease-cleavage sites (Tamberg et al., 2007). Similarly, a Renilla luciferase (Rluc) gene was inserted into our CHIKV Mauritian clone between the nsP3 and nsP4 coding regions (Bouraï et al., 2012). In this case, the Rluc was flanked at its N terminus by the nsP1/2 protease recognition site of CHIKV Mauritian and C-terminally by the nsP3/4 protease recognition site (Bouraï et al., 2012). For CHIKV (La Reunion isolate), both replicon and full-length virus were described encoding a Rluc marker introduced after aa 490 within nsP3 (Pohjala et al., 2011). Hence, we planned to test this insertion site based on our newly established infectious CHIKV Mauritian full-length clone for the expression of a fluorescent reporter. In addition, a site further upstream in CHIKV nsP3 corresponding to a region previously demonstrated to yield replication competent SINV upon Rluc and EGFP insertion (Bick et al., 2003; Frolova et al., 2006) should be tested and compared as well. The respective insertion site corresponds to aa 377 of CHIKV nsP3, resulting in an mCherry protein inserted between histidine 377 and threonine 378 of nsP3.

With regard to fluorescent proteins, GFP has been the gold standard over the past decade. However, GFP has an emission spectrum overlapping with many endogenous proteins, resulting in background fluorescence. In particular, green autofluorescence has been observed in certain insects, especially in the thorax (Stefanie Müller, Hamburg, Germany, personal communication). To circumvent this problem, proteins with longer emission wavelength like red fluorescent proteins can be used. Comparison of RedStar, tdTomato and mCherry revealed that tdTomato bleaches almost as rapidly as RedStar, whereas mCherry exhibits improved photostability (Graewe et al., 2009). Therefore, the latter protein was used for the construction of our reporter gene expressing rCHIKV.

The mCherry gene was amplified from pmCherry-N1 (Clontech). Depending on the insertion site either after codon 377 or codon 490 of the nsP3 gene, it was fused to the appropriate flanking sequences of CHIKV using fusion PCR technology (Charlier et al., 2003). The resulting fusion cassettes were cut with suitable restriction enzymes and each one was introduced into pCHIKV-M using three fragment ligation reactions. The obtained constructs were designated pCHIKV-mCherry-377 or pCHIKV-mCherry-490, respectively (Fig. 3a).

After electroporation of in vitro transcripts derived from the NcoI linearized plasmids into BHK cells, specific infectivities comparable to the one obtained for the unmodified rCHIKV were obtained by infectious centre assay (Fig. 3b). In addition, the plaques were similar in size (Fig. 3b). To confirm the expression of mCherry, virus harvested at 48 h post-electroporation was used to reinfect fresh BHK cells at an m.o.i. of 0.1. At 12 h post-infection (p.i.), cells clearly showed red fluorescence (Fig. 3c). Furthermore, punctate fluorescence could be observed as was described before to be typical for nsP3 localization (Varjak et al., 2010). In addition, the mCherry autofluorescence is retained inside the fixed cells after permeabilization. Hence, using anti-dsRNA antibody (mouse mAb J2; English & Scientific Consulting Bt.) dual localization of the viral replication complexes and the chimeric nsP3–mCherry protein could be performed. As can be seen in Fig. 3(d), a high number of the nsP3 containing structures also contained dsRNA, similar to what was described for SINV (Gorchakov et al., 2008).

To compare the growth characteristics of the reporter viruses in further detail, BHK and C6/36 cells were infected at an m.o.i. of 0.1 with the mCherry expressing viruses and compared to the unmodified rCHIKV (Fig. 3e). Similar growth on both BHK and C6/36 cells already showed that the mCherry insertions did not dramatically affect the fitness of the viruses, suggesting stable replication of the reporter viruses. To further analyse the stability of the mCherry
reporter viruses, they were passaged four times at an m.o.i. of 0.1 on BHK cells. After the fourth passage, 10 plaques each were picked. Infection of fresh cells with the plaque-purified viruses resulted in all cases in mCherry expression, suggesting the stable replication of the mCherry reporter viruses in BHK cells (data not shown). In addition, the mCherry viruses were also passaged four times on C6/36 cells (m.o.i. of 0.001) and thereafter analysed for red fluorescence either directly or after plaque purification on BHK cells. For both mCherry reporter viruses, red fluorescence was also retained after four passages on the C6/36 cells (data not shown). Therefore, besides the already described insertion site after aa position 490 of CHIKV nsP3 (Pohjala et al., 2011), aa position 377 of CHIKV nsP3 also constitutes a suitable insertion site.

The established mCherry reporter viruses represent a valuable tool for studies involving fluorescent CHIKV, especially in cases where high background using a FITC filter is observed or in addition to EGFP expression for dual-infection experiments.

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