Characterization of an infectious clone of the wild-type yellow fever virus Asibi strain that is able to infect and disseminate in mosquitoes

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Infectious clone technology provides an opportunity to study the molecular basis of arthropod–virus interactions in detail. This study describes the development of an infectious clone of the prototype yellow fever virus Asibi strain (YFV-As) with the purpose of identifying sequences or domains that influence infection dynamics in the mosquito vector. The full-length cDNA of YFV-As virus was produced from RT-PCR products of parental viral RNA. These were cloned into a low-copy-number plasmid previously used to develop the YFV-17D infectious clone (pACNR/FLYF-17D). Virus recovered from the infectious clone exhibited biological characteristics similar to those of the parental YFV-As, including replication kinetics, reactivity to flavivirus cross-reactive and YFV-specific antibodies and infection and dissemination rates in Aedes aegypti, the principal mosquito vector of YFV. These data provide the basis for future studies with chimeric Asibi/17D viruses to identify the determinants of vaccine attenuation in the vector.

Yellow fever virus (YFV) was the first mosquito-borne virus to be identified (Stokes et al., 1928). It causes a severe and often fatal haemorrhagic disease in humans, for which there is no specific treatment. Despite the existence of two safe and efficacious vaccines, YFV 17D-204 and 17DD, the incidence of YFV infections is increasing, and the disease has re-emerged in many parts of Africa and South America where incidence had previously been reduced largely through mosquito control programmes (Barrett & Monath, 2003; Mutebi & Barrett, 2002; Robertson et al., 1996). The remoteness of many regions in which YFV occurs hinders accurate data collection, but it is estimated that there are 200 000 infections and 30 000 deaths annually.

YFV is the prototype member of the genus Flavivirus, family Flaviviridae, which includes other important human pathogens, for example, dengue viruses (DENV), West Nile virus (WNV), Japanese encephalitis virus (JEV) and tick-borne encephalitis virus (TBEV). The prototype YFV strain, Asibi (Ghana27), was isolated from Mr Asibi in 1927 (Stokes et al., 1928). Flaviviruses are single-stranded positive-sense RNA viruses with genomes of approximately 11 kb that encode three structural proteins, capsid, membrane and envelope, and seven non-structural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5, between 5’ and 3’ non-coding regions (NCRs). Virus is transcribed and translated as one long polyprotein precursor that is processed co- and post-translationally by host and viral proteases (Lindenbach & Rice, 2003).

Infectious cDNA clones provide a useful platform on which to investigate the genetic determinants of flavivirus virulence. Initially, flavivirus infectious clones, including one for YFV (Rice et al., 1989), depended on a two-plasmid system with in vitro ligation. However, a full-length YFV infectious clone, pACNR/FLYF-17D, based on YFV 17D-204, was recently described (Bredenbeek et al., 2003). Infectious clones based on virulent virus strains and the capacity to generate chimeric viruses with components of virulent and attenuated strains could greatly facilitate elucidation of the molecular determinants of virulence and identify targets for potential therapies. The YFV Asibi strain (YFV-As) was chosen for these studies for several reasons: it is the prototype YFV strain and it is the strain from which the 17D vaccines were derived. It also infects and disseminates efficiently in Aedes (Ae.) aegypti mosquitoes (Hahn et al., 1987; Miller & Adkins, 1988; Miller & Mitchell, 1991), whereas the YFV 17D strain is able to infect but not to disseminate in Ae. aegypti (Jennings et al., 1994; Miller & Adkins, 1988; Whitman, 1939).

YFV-As was obtained from the World Reference Center for Arboviruses at the University of Texas Medical Branch (UTMB), Galveston, TX, USA. This virus had been passed six times in monkeys and twice in C6/36 (Ae. albopictus) cell
culture since its original isolation. Stock virus was produced following a single additional passage in C6/36 cells (m.o.i. = 0.01) grown at 28°C in Leibovitz L-15 medium with 10% FBS and 100 U penicillin and 100 μg streptomycin ml⁻¹. Virus was harvested from supernatant medium when cells showed 75% cytopathic effect. Aliquots were stored at −80°C for use in all experiments. Viral RNA was extracted from an aliquot using the QIAamp Viral RNA Mini kit (Qiagen) following the manufacturer's protocol and stored at −80°C. All manipulations of live virus were performed under Biosafety Level 3 conditions.

The YFV 17D-204 infectious clone (pACNR/FLYF-17D; Bredenbeek et al., 2003) was used as the cloning vector for the construction of the YFV-As infectious clone (YFV-As ic) to utilize the low-copy-number plasmid pACNR1181 present in the infectious clone. All plasmids used for the construction of YFV-As ic were prepared by standard PCR-based mutagenesis and cloning methods (Sambrook et al., 1989) and all regions amplified by PCR were verified by direct sequence analysis at the UTMB Protein Chemistry Laboratory. RT-PCR of the Asibi RNA to produce cDNA was performed using random hexanucleotide primers (Promega) and Superscript II (Invitrogen Life Technologies) following the manufacturer’s protocol, and cDNA was amplified with Pfu DNA polymerase (Stratagene). Reverse transcription of the 3′ NCR was performed using the Titan One Step RT-PCR kit (Roche). Since the nucleotide sequence of the 5′ NCR and the amino acid sequence of the C gene of Asibi virus are identical to that of 17D-204, this region was not amplified. Eighteen primers were used to generate the cDNA fragments and to determine the YFV-As ic sequence. All plasmids were extracted using the QIAprep Spin Miniprep kit (Qiagen) following the manufacturer’s protocol, and cDNA was ligated with T4 DNA ligase (Invitrogen Life Technologies), creating four fragments, as shown in Fig. 1. They were then cloned sequentially into pACNR/FLYF-17D, replacing the equivalent fragment of the 17D cDNA. Full-length Asibi DNA was amplified in MC1061 cells. The sequence of the cDNA was determined following assembly of all fragments into the full-length clone. Sequence differences were observed between the YFV-As ic used in these studies and the YFV-As parent virus from which it was derived (Table 1). Five silent nucleotides and three substitutions resulting in amino acid differences were detected between YFV-As ic and YFV-As.

To produce infectious Asibi virus, the YFV-As ic plasmid was linearized with NruI, purified with phenol/chloroform and in vitro-transcribed using the SP6 mMESSAGE mMACHINE capped RNA transcription kit (Ambion). For virus production and measurement of specific infectivity, 10 μg RNA was electroporated into BHK-21 cells in L-15 as described previously (Higgs et al., 1997) using the Gene Pulser Xcell electroporation system (Bio-Rad). The specific infectivity of RNA from YFV-As ic, measured by infectious centre assay in Vero cells following a protocol reported previously by Yun et al. (2003), was 1.6 × 10⁹ f.f.u. (μg RNA)⁻¹. Virus replication was monitored every 24 h in BHK-21 cells after electroporation by titration of virus recovered from the cell culture supernatant medium and inoculated onto Vero cells in L-15 medium. Indirect immunofluorescence assay (IFA) of the electroporated BHK cells was performed as described previously (Gould et al., 1985; Higgs et al., 1997; Vanlandingham et al., 2005) using a YFV-reactive polyclonal antibody, MA93, diluted 1:500 in PBS. For all IFA, signal amplification was achieved by using a biotinylated secondary antibody and streptavidin-conjugated fluorescein diluted 1:200 in PBS. All cells were examined under UV light with a fluorescein filter using an Olympus IX-70 epifluorescence microscope. Viral titre increased rapidly in BHK cells and peaked at 4 days post-electroporation at 8.5 log₁₀ TCID₅₀ ml⁻¹ (Fig. 2a), corresponding to virus spread as determined by daily antigen detection by IFA (data not shown).

![Fig. 1. Construction of pYFV-As ic. Nucleotide positions and unique restriction sites used for cloning are indicated.](image-url)

**Table 1. Sequence differences between the YFV-As parent virus and YFV-As ic**

<table>
<thead>
<tr>
<th>Position</th>
<th>YFV-As nt</th>
<th>YFV-As ic nt</th>
<th>Amino acid position</th>
</tr>
</thead>
<tbody>
<tr>
<td>1819</td>
<td>C/U</td>
<td>S/F</td>
<td>E₂₈₂</td>
</tr>
<tr>
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<td>C/U</td>
<td>A/V</td>
<td>E₄₀₇</td>
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<tr>
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<td>A/G</td>
<td>K/R</td>
<td>NS₁₀</td>
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<td>E</td>
<td>A</td>
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<td>U</td>
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</tr>
<tr>
<td>7642</td>
<td>C/U</td>
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</table>

*No sequence difference at this position between YFV-As ic and pACNR/FLYF-17D.*
Comparative growth curves for YFV-As and the YFV-As ic virus in C6/36 cells following infection at m.o.i. 0·01 were generated by estimating the mean infectivity of virus recovered every 24 h from the supernatant medium of two independent experiments. These values were expressed as log_{10} TCID_{50} ml^{-1} (Fig. 2b). No significant differences were observed at any time point between the replication of YFV-As ic and YFV-As in C6/36 cell culture.

Additionally, the YFV-As ic was characterized and compared with wild-type YFV-As and virus derived from pACNR/FLFY-17D using a panel of antibodies: MA93 (YFV hyperimmune antibody), mAb 813 (broadly flavivirus-reactive), mAb 117 (YFV wild-type-specific) and mAb 864 (YFV 17D-specific) (Gould et al., 1985). Uninfected Vero cells were used as negative controls. As anticipated, cells infected with YFV-As ic were detected by MA93, mAb 813 and mAb 117 but not by mAb 864, which is specific for the 17D vaccine. This is identical to the reaction profile seen with the parent Asibi virus. Virus derived from the YFV 17D infectious clone was visualized by all of the antibodies except the wild-type-specific mAb 117.

Colonized Ae. aegypti mosquitoes, RexD strain, obtained from Rexville Puerto Rico, of known susceptibility to infection by YFV (Miller & Mitchell, 1991), were used to determine whether or not YFV-As ic could infect mosquitoes when administered orally. Infectious YFV-As ic virus and YFV-As virus were each mixed with an equal volume of defibrinated sheep blood and 3 mM ATP was added as a phagostimulant. This infectious blood meal was pipetted into the chamber of a membrane feeding system (Haemotek). Five-day-old mosquitoes [100 per 1 pint (473 ml) carton] were starved for 24 h and allowed to feed on the infectious blood meal for 1 h. Samples of the blood meal were taken and frozen at −80 °C for analysis. Replete females were returned to the carton and held at 28 °C for 14 days. To determine the percentage of blood-fed mosquitoes that developed a disseminated infection, salivary glands were dissected onto microslides, dried, fixed in acetone and stained by IFA as described above. Carcasses were frozen at −80 °C for subsequent analysis of virus infectivity by titration on Vero cells to determine the percentage infected as described previously (Higgs et al., 1997).

In the case of YFV-As ic, titration confirmed that blood meals in both experiments contained 6·5 log_{10} TCID_{50} ml^{-1} of virus, and the virus titre in day 0 mosquitoes was 4·0 ± 0·46 log_{10} TCID_{50} ml^{-1}. At 14 days post-infection (p.i.), the total mean titre recovered from whole mosquitoes was 3·63 ± 1·17 log_{10} TCID_{50} ml^{-1} and titration of carcasses showed that 42/58 mosquitoes (72%) were infected, whilst IFA of salivary glands indicated a dissemination rate of 83% (35/42). For YFV-As parent virus, the blood meals contained 6·95 and 7·52 log_{10} TCID_{50} ml^{-1} virus, respectively, and the mean day 0 whole mosquito titre was 3·99 ± 0·91 log_{10} TCID_{50} ml^{-1}. At 14 days p.i., the mean titre recovered from whole mosquitoes was 3·34 ± 1·36 log_{10} TCID_{50} ml^{-1} and 73% (24/33) of mosquitoes were infected, whilst 75% of these (18/24) had disseminated infections as determined by IFA. The differences in titre between virus recovered from whole mosquitoes and the infection and dissemination rates of YFV-As ic compared with the parental YFV-As are not statistically significant as determined by Fisher’s exact test.

Infectious clones facilitate detailed molecular and biological studies of virus replication, structure, antigenicity, virulence determinants and vaccine development. The first full-length flavivirus infectious clones were developed using two-plasmid constructs which were ligated in vitro. This approach was used for YFV (Rice et al., 1989), DENV-2 (Kapoor et al., 1995), JEV (Sumiyoshi et al., 1992) and TBEV (Gritsun & Gould, 1998; Mandl et al., 1997). However, many of these clones were unstable. Therefore, through the use of low-copy-number vector plasmids and specific bacterial host cells, a number of stable full-length flavivirus infectious clones have been developed and reported, including those for DENV (Lai et al., 1991; Kinney et al., 1997), TBEV (Gritsun & Gould, 1998; Hayasaka et al., 2004; Mandl et al., 1997), WNV (Shi et al., 2002; Yamshchikov et al., 2001) and JEV (Yun et al., 2003). With the exception of differences in cell culture replication kinetics reported for a TBEV infectious clone (Hayasaka et al., 2004), the
resulting infectious clones were stable, and virus produced from these infectious clones retained the biological and phenotypic characteristics of their parental viruses.

In our work, the use of the low-copy-number plasmid pACNR1181 for insertion of YFV-As-derived cDNA fragments facilitated the rapid development of a stable full-length YFV-As virus infectious clone. Virus derived from the infectious clone replicated as efficiently as parental virus when they were compared in C6/36 cells and virus derived from the infectious clone was antigenically indistinguishable from the parent Asibi virus as determined by using a limited panel of antibodies.

Despite our extensive knowledge concerning the transmission cycles of various arboviruses (Higgs, 2004), the viral genetic determinants of vector competence remain unclear. Perhaps the most important observation of our studies at this stage was the fact that virus derived from the infectious clone infected Ae. aegypti mosquitoes when administered orally. Moreover, this virus replicated to comparable titres by day 14 p.i. and was capable of producing a disseminated infection at a rate comparable to that of the parent virus. The high rates of infection and dissemination following exposure to YFV-As ic demonstrate retention of the parental phenotype following oral infection. Thus, comparison of infectious clones based on the disseminating wild-type virus YFV-As and the non-disseminating, attenuated virus YFV 17D provides an ideal model system to identify the genetic basis of virus transmission by mosquito vectors. These studies are currently under way using YFV-As ic. Information gained using this infectious clone may then be applied to related flaviviruses such as DENV and WNV to ensure that live virus vaccine candidates are not able to be transmitted by potential mosquito vectors.

The YFV-As strain upon which the present infectious clone is based was originally subcultured 176 times to produce the 17D virus (Theiler & Smith, 1937). The resulting virus lost the ability to cause viscerotropic disease and to disseminate in mosquitoes but retained its immunogenicity when inoculated into monkeys and humans. This virus formed the basis for the vaccines 17DD and 17D-204 (Theiler, 1951) which are still being used today and have successfully immunized millions of humans. Sequence comparisons between Asibi and 17D provided an ideal model system to identify the genetic basis of virus transmission by mosquito vectors. These studies are currently under way using YFV-As ic. Information gained using this infectious clone may then be applied to related flaviviruses such as DENV and WNV to ensure that live virus vaccine candidates are not able to be transmitted by potential mosquito vectors.

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References


