Identification of new flaviviruses in the Kokobera virus complex

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Novel flavivirus isolates from mosquitoes collected in northern Australia were analysed by partial genomic sequencing, monoclonal antibody-binding assays and polyclonal cross-neutralization tests. Two isolates were found to be antigenically distinct from, but related to, viruses of the Kokobera virus complex, which currently contains Kokobera (KOKV) and Stratford (STRV) viruses. Nucleotide sequence comparison of two separate regions of the genome revealed that an isolate from Saibai Island in the Torres Strait in 2000 (TS5273) was related closely to KOKV and STRV, with 74–80 and 75–76 % nucleotide similarity, respectively. An isolate from mainland Cape York in 1998 (CY1014) was found to be more divergent from KOKV and STRV, with <70 % nucleotide sequence similarity to either virus. It is proposed that isolate TS5273 represents a new subtype of KOKV and that CY1014 be classified as a novel species within the Kokobera virus complex of flaviviruses, named New Mapoon virus.

Kokobera virus (KOKV) is a mosquito-borne flavivirus that has been isolated from mosquitoes throughout Australia and Papua New Guinea (PNG) (Mackenzie et al., 1994). It was originally isolated from Culex annulirostris mosquitoes collected at Kowanyama (Mitchell River Mission) in northern Queensland in 1960 and was named after a local Aboriginal tribe (Doherty et al., 1963). Since that time, KOKV has been isolated from mosquitoes collected in Western Australia, Northern Territory, New South Wales (NSW) and Queensland (QLD) (Russell, 1995). Serological evidence suggests that macropods (kangaroos and wallabies) and horses may be reservoir hosts of KOKV (Doherty et al., 1964, 1971). Human infections with KOKV occasionally result in an acute polyarticular disease (Doherty et al., 1964; Hawkes et al., 1985, 1993; Boughton et al., 1986).

KOKV and the closely related Stratford virus (STRV) were originally classified as separate species in the Japanese encephalitis virus (JEV) complex within the genus Flavivirus. Important members of this complex include JEV, Murray Valley encephalitis virus (MVEV), West Nile virus and Saint Louis encephalitis virus (Mackenzie et al., 2002). However, recent studies have indicated that although KOKV and STRV are related closely to each other, they are antigenically and genetically distinct from the other viruses in the JEV group (Hall et al., 1991; Poidinger et al., 1996; Kuno et al., 1998). This has led to the reclassification of KOKV into a separate virus complex within the genus, with STRV listed as a subtype of KOKV (Heinz et al., 2000). This paper describes the characterization of two novel flaviviruses in the Kokobera virus complex and examines their phylogenetic relationship with other viruses within the genus Flavivirus.

Virus isolates were obtained from mosquitoes collected in 1998 on Cape York Peninsula and in 2000 on Saibai Island, as described previously (van den Hurk et al., 2001; Johansen et al., 2003, 2004). Virus stocks were prepared by inoculating confluent monolayers of PS-EK cells (Gorman et al., 1975) and harvesting culture supernatants when at least 70 % of the cells exhibited cytopathic effect.

Six isolates were initially identified as KOKV based on their reaction with a panel of monoclonal antibodies (mAbs) in

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are available as supplementary material in JGV Online.
an ELISA (Broom et al., 1998). Further investigations with a panel of non-neutralizing mAbs prepared to the E and NS1 proteins of KOKV isolate MRM32 (Hall et al., 1991) revealed that four isolates showed an identical mAb-binding profile to that of KOKV MRM32 (Table 1). However, one isolate (TS5273) was recognized by only five of the seven mAbs, showing a similar binding pattern to that reported previously for a genetically divergent KOKV isolate from PNG (MK7979) (Poidinger et al., 2000). A second isolate (CY1014) and the prototype STRV strain (C338) also showed distinct binding patterns, the former being recognized by only three of the seven mAbs and the latter by only two (Table 1).

To further investigate the antigenic differences between the two novel isolates and the prototype strains of KOKV and STRV, cross-neutralization studies were performed. Hyperimmune mouse sera were produced to KOKV MRM32, STRV C338, CY1014 and TS5273 in 7-week-old BALB/c mice by intraperitoneal (i.p.) injection at 2-week intervals with three doses of 1000 infectious units (IU) of virus only, 1 dose of 10 000 IU with 50 % adjuvant (MPL + TDM Emulsion adjuvant; Sigma) i.p. and a final subcutaneous immunization of 100 000 IU in 50 % adjuvant. Once significant neutralization titres (>80) to the homologous virus were achieved, mice were exsanguinated by cardiac puncture. Pooled sera from each mouse group were tested for homologous and heterologous neutralization as described previously (Hall et al., 1987) (Table 2). Both STRV C338 and KOKV MRM32 antisera clearly neutralized homologous virus, with at least a fourfold difference in titre when compared with other viruses. TS5273 antisera also discriminated between homologous virus and KOKV MRM32, but not between TS5273 and STRV C338, implying a closer antigenic relationship between the latter two viruses. The flavivirus group-reactive mAb 4G2, obtained from the ATCC (catalogue no. HB-112; Gentry et al., 1982) was included as a positive control, as it has previously been shown to neutralize both STRV C338 and KOKV MRM32 (Hall et al., 1987). However, in this study, 4G2 efficiently neutralized only STRV C338, differentiating it from KOKV MRM32 and TS5273. Neutralizing titres to CY1014 could not be determined, as it failed to produce distinct cytopathic effect in PS-EK cells.

To determine the genetic relationship of the novel KOKV-like isolates to KOKV MRM32 and STRV C338, viral RNA was extracted from infected PS-EK cells and RT-PCR was performed as described previously (Johansen et al., 2000), using conserved flavivirus primers VD8 and EMF (Pierre

### Table 1. Reaction of mAbs to KOKV, STRV and Kokobera-like viruses in ELISA

ELISA was performed as described by Broom et al. (1998).

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Location and year of isolation</th>
<th>mAb*</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOKV MRM32</td>
<td>Kowanyama, QLD, 1960</td>
<td>++</td>
</tr>
<tr>
<td>CY1014</td>
<td>New Mapoon, QLD, 1998</td>
<td>--</td>
</tr>
<tr>
<td>CY1026</td>
<td>Seisia, QLD, 1998</td>
<td>+</td>
</tr>
<tr>
<td>CY1049</td>
<td>Seisia, QLD, 1998</td>
<td>+</td>
</tr>
<tr>
<td>CY1051</td>
<td>Seisia, QLD, 1998</td>
<td>+</td>
</tr>
<tr>
<td>CY1053</td>
<td>Seisia, QLD, 1998</td>
<td>+</td>
</tr>
<tr>
<td>TS5273</td>
<td>Saibai Island, QLD, 2000</td>
<td>+</td>
</tr>
<tr>
<td>STRV C338</td>
<td>Cairns, QLD, 1961</td>
<td>--</td>
</tr>
<tr>
<td>MK7979†</td>
<td>Sepik, PNG, 1966</td>
<td>++</td>
</tr>
<tr>
<td>WD26547†</td>
<td>Wandoona, NSW, 1981</td>
<td>+</td>
</tr>
</tbody>
</table>

* mAbs were produced to the MRM32 strain of KOKV virus (see Hall et al., 1991).
† Data published by Poidinger et al. (2000).

### Table 2. Heterologous and homologous neutralization titres of antisera to KOKV, STRV and TS5273 in a microneutralization assay

Assay performed as described by Hall et al. (1987).

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Virus strain</th>
<th>KOKV MRM32</th>
<th>STRV C338</th>
<th>TS5273</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-KOKV MRM32</td>
<td>80*</td>
<td>&lt;20</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td>α-STRV C338</td>
<td>20</td>
<td>80</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td>α-TS5273</td>
<td>80</td>
<td>320</td>
<td>320</td>
<td></td>
</tr>
<tr>
<td>4G2†</td>
<td>&lt;10</td>
<td>160</td>
<td>&lt;10</td>
<td></td>
</tr>
</tbody>
</table>

* Neutralization titre determined as the reciprocal of the highest serum dilution to produce 80 % reduction of cytopathic effect in PS-EK cells.
† Group-reactive flavivirus mAb (see Gentry et al., 1982).
et al., 1994; Poidinger et al., 1996). These primers amplify a region extending from the 3’ end of the NS5 gene to the 5’ end of the 3’ untranslated region (UTR) of the flavivirus genome. DNA products from the PCRs were purified and sequenced by using the BigDye system (Perkin Elmer, Applied Biosystems). Sequence of a 521 nt region (beginning at the nucleotide corresponding to position 10131 in the KUNV genome; Coia et al., 1988) was obtained for all isolates in the NS5/3’ UTR and aligned with previously published nucleotide sequences of other KOKV and STRV strains (Poidinger et al., 2000) by using CLUSTAL W (Thompson et al., 1994) on BioManager [Australian National Genomic Information Service (ANGIS) (Sydney, Australia)]. A table of GenBank accession numbers for the sequences used in this paper is available as supplementary material in JGV Online. All isolates from Cape York, except CY1014, shared 100 % nucleotide similarity with each other and 96 % with the prototype KOKV MRM32. The nucleotide sequence alignment revealed that CY1014 displayed only 67 % similarity to the KOKV prototype MRM32, 69 % to STRV C338 and 69 % to TS5273. In comparison, TS5273 exhibited 75 % nucleotide similarity to KOKV MRM32 and 80 % to STRV C338. Although TS5273 displayed the same mAb binding profile as the PNG KOKV isolate MK7979, nucleotide sequencing revealed only 75 % nucleotide similarity between these viruses. These results confirmed the results of the antigenic studies, indicating that CY1014 and, to a lesser extent, TS5273, were distinct from KOKV and STRV.

To determine their relationship to other flaviviruses, CY1014 and TS5273 were also sequenced by using the primer pair cFD3 and FU1 (Kuno et al., 1998) or a degenerate version of these primers (cFD3PM and FU1PM). These primers amplify approximately 1 kb of the NS5 gene, using the same conditions as described above. Multiple sequence alignments of a 677 nt sequence (corresponding to nt 9312–9988 of the KUNV genome; Coia et al., 1988) of CY1014, TS5273 and previously published nucleotide sequences of this region from a range of flaviviruses (Kuno et al., 1998) were performed by using CLUSTAL W. This analysis confirmed that CY1014 was related most closely to KOKV, but shared only 69 % similarity with this virus and 67 % with STRV. In contrast to the sequence alignments obtained by using the EMF/VD8 primers, nucleotide sequence from the cFD3 and FU1 region of NS5 revealed that TS5273 had a slightly closer relationship to KOKV (76 % nucleotide similarity) than to STRV (74 % nucleotide similarity). However, TS5273 showed a closer relationship to STRV in the deduced amino acid sequence of this region (94 %) than to KOKV (91 %).

Phylogenetic analysis was then performed on multiple sequence alignments of nucleotide sequence obtained from the cFD3 and FU1 region by using the PHYLIP package (Felsenstein, 1989) and a phylogram was created by using the neighbour-joining method (Fig. 1). Confidence values were placed on branches by using bootstrap analysis of 100 replicates. The tree reveals that CY1014 branches with KOKV, STRV and TS5273, but is the most evolutionary distant member of the group. TS5273, on the other hand, clusters with STRV, but the bootstrap value is very low. This reflects the equivocal data that were obtained from both antigenic studies and nucleotide sequencing, some of which suggest a closer relationship between TS5273 and STRV (cross-neutralization, EMF/VD8 nucleotide sequence and cFD3 and FU1 amino acid sequence) and some indicate higher similarity of TS5273 to KOKV (mAb binding and cFD3 and FU1 nucleotide sequence). In summary, it can be concluded that TS5273 is closely related to, but distinct from, both KOKV and its subtype STRV.

The comprehensive study on the phylogeny of flaviviruses described by Kuno et al. (1998) proposed that a species was a set of viruses with > 84 % nucleotide similarity and a clade comprised a set of viruses with > 69 % similarity. Based on these criteria and the results described above, CY1014 should be classified as a novel species within the KOKV complex. We propose the name New Mapoon virus, after the location where it was isolated. Our results also indicate that isolate TS5273 has a closer relationship to both KOKV and STRV and should probably be considered as a new subtype of KOKV.

The isolation of two novel viruses from northern Australia was unexpected, given the considerable effort expended on surveillance for mosquito-borne viruses throughout Australia over many years. Whether they were always present, perhaps in cryptic foci or in areas or niches that were not investigated previously, or whether they were introduced, is unknown.
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References


