Isolation and complete nucleotide sequence of a Chinese Sindbis-like virus

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Infection with alphaviruses is common in the Chinese population. Here we report the isolation of a Sindbis-like virus from a pool of Anopheles mosquitoes collected in Xinjiang, China during an arbovirus survey. This virus, designated XJ-160, rapidly produced cytopathic effects on mosquito and hamster cells. In addition, it was lethal to neonatal mice if inoculated intracerebrally. Serologically, XJ-160 reacted with and was neutralized by an anti-Sindbis antibody. Anti-XJ-160 antibodies were found in several cohorts of Chinese subjects. The complete 11626-base nucleotide sequence of XJ-160 was determined. XJ-160 has diverged significantly from the prototype Sindbis virus, with an 18% difference in nucleotide sequence and an 8.6% difference in amino acids; there are 11 deletions and 2 insertions, involving 99 nucleotides in total. XJ-160 is most closely linked to Kyzylagach virus isolated in Azerbaijan. Both belong to the African/European genetic lineage of Sindbis virus, albeit more distantly related to other members.

Arboviruses are transmitted by blood-sucking insects. Globally, arboviral infections are a significant cause of morbidity and mortality in humans and in animals. However, of more than 500 arboviruses recognized worldwide, only four (Japanese encephalitis virus, Dengue virus, Xinjiang haemorrhagic fever virus and Russian spring–summer encephalitis virus) have been unambiguously found in China (Chen & Tao, 1996). In order to identify more Chinese strains of arboviruses, we conducted an arbovirus survey in China. During this survey, we isolated two arboviruses, designated XJ-160 and YN87448. YN87448 was isolated in 1992 from a serum sample collected from a Chinese patient suffering from a febrile illness. The patient was from Yunan, China. YN87448 has been characterized as a Sindbis virus most closely related to the Girdwood S. A. strain. More details of YN87448 will be published later (G.-D. Liang, unpublished data). Here we report the isolation and complete nucleotide sequence of XJ-160.

XJ-160 was recovered in 1990 from a pooled sample of Anopheles mosquitoes collected in a rice field along the Yili River in Xinjiang, China. The captured mosquitoes were kept in liquid nitrogen and sent to the laboratory where the virus was isolated. The mosquito tissues were ground up and the supernatant was inoculated onto C6/36 mosquito cells. Cytopathic effects (CPE) were seen within 72 h. The infected cells rounded out, shrank and peeled off. The supernatant of infected C6/36 cells also produced CPE in BHK-21 hamster cells within 48 h. Neonatal (> 2 days old) mice injected intracerebrally with XJ-160 died within 48 h; however, intracerebral inoculation of XJ-160 was not lethal to 3-week-old mice.

Under the electron microscope, XJ-160 appeared as spherical particles with a diameter of 58.7 ± 2.3 nm (n = 10). A dense viral nucleocapsid was seen enclosed within an envelope with arrays of radiated spikes (data not shown). XJ-160 was sensitive to acid (pH 3–0; treated for 2 h), heat (56 °C for 30 min), ether (20% for 18 h) or ultraviolet light (for 15 min), but resistant to 5-fluorodeoxyuridine (50 μg/ml).

The biological and physico-chemical characteristics described above suggested that XJ-160 might be a togavirus. Thus we performed serological tests using the XJ-160 virus and the anti-XJ-160 immune ascitic fluids. In ELISA, XJ-160 reacted strongly with both anti-Sindbis and anti-chikungunya, but not with antibodies against any of the following viruses: Eastern equine encephalitis virus, Western equine encephalitis virus, Semliki Forest virus, Sagiyma virus, Mayaro virus, getah virus, Japanese encephalitis virus, Dengue virus serotypes 1–4, Kunjin virus, Murray Valley encephalitis virus, West Nile virus, Powassan virus, Bunyamwera virus, California enceph-
Alphavirus in the family Liang, 1997). Sporadically reported in the same areas (Chen & Tao, 1996; was originally isolated from Yili area. It remains open that XJ-160 was cross-neutralized by anti-Sindbis and vice versa (Table 1). Thus we conclude that XJ-160 is a Sindbis-like virus. It is noteworthy that this is the first Chinese isolate of a Sindbis-like virus. Previously, we have shown that infection with alphaviruses is rather common in Chinese (Chen et al., 1983). To further characterize XJ-160, we performed a neutralization assay in neonatal mice (Table 1). As expected, each of the four viruses (XJ-160, Sindbis, chikungunya and Eastern equine encephalitis) was neutralized individually by specific antibodies (i.e. XJ-160 was neutralized by anti-XJ-160, etc.). In addition, XJ-160 was cross-neutralized by anti-Sindbis and vice versa (Table 1). Thus we conclude that XJ-160 is a Sindbis-like virus. It is noteworthy that this is the first Chinese isolate of a Sindbis-like virus. Previously, we have shown that infection with alphaviruses is rather common in Chinese (Chen et al., 1983). To further understand the sero-prevalence of XJ-160, we carried out an epidemiological survey of anti-XJ-160 antibodies. Of 521 subjects from 10 different provinces of China, 99 were positive for anti-XJ-160. Notably, the positivity of subjects from the following three areas was significantly higher: Ningxia (54.3%), Anhui (40.0%) and Yili area, Xinjiang (31.5%). XJ-160 was originally isolated from Yili area. It remains open that XJ-160 might be aetiologically associated with the febrile illness sporadically reported in the same areas (Chen & Tao, 1996; Liang, 1997).

Sindbis virus is the prototype species of the genus Alphavirus in the family Togaviridae (Strauss & Strauss, 1994). Sindbis-like viruses have a wide geographical distribution (Olson & Trent, 1985; Norder et al., 1996; Sammels et al., 1999) and many of them can cause human diseases (Mackenzie et al., 1994; Simpson et al., 1996). Currently, the complete genome sequences of six strains of Sindbis-like viruses have been deposited in GenBank. AR339 represents the original strain of Sindbis virus isolated from Sindbis village in Egypt, whereas HRsp is the small-plaque variant of the heat-resistant strain derived from AR339 (Strauss et al., 1984; McKnight et al., 1996). Ockelbo is a disease-causing strain first isolated in Sweden (Shirako et al., 1991). S.A. AR86 is a South African strain temporarily associated with human disease (Simpson et al., 1996). Girdwood S.A. is also a South African strain, but it was isolated from a human patient (Simpson et al., 1996). YN87448 is significantly homologous to Girdwood S.A., but it was isolated from a Chinese patient (G.-D. Liang, unpublished data; GenBank no. AF103734). Aura is a strain found in Brazil and Argentina (Kumenapf et al., 1995). It is more diverged from AR339 than all other strains are. Notably, none of the above strains except YN87448 is from Asia.

Previously, two distinct genetic lineages (African/European and Asian/Australian) of Sindbis-like virus have been defined on the basis of RNA–RNA hybridization, RNase T1 mapping and antigenic analysis (Rentier-Delrue & Young, 1980; Olson & Trent, 1985). A more recent analysis of nucleotide sequence data from 40 Sindbis-like virus isolates supports the separation of the two lineages (Sammels et al., 1999). It would be of interest to see whether XJ-160 is more closely related to the Asian/Australian strains.

In order to study the molecular evolution of the Chinese XJ-160 virus, we sought to determine its complete nucleotide sequence. XJ-160 genomic RNA was isolated from the brain of infected mice and RT–PCR was performed as described (Wang & Jin, 1997; Wang et al., 1997a, b). Degenerate primers were used to amplify 15 overlapping fragments. The 5′-terminal sequence was obtained by the 5′-RACE (rapid amplification of cDNA ends) procedure. In order to resolve discrepancies, three more independent fragments were generated. All fragments were cloned and sequenced from both directions. Details of primers and strategies for cloning and sequencing are available upon request.

The complete genomic sequence of XJ-160 contained 11626 nt, encoding 3731 amino acids. The non-structural region comprised 7461 nt (60–7523), encoding four non-structural proteins (nsP1–4; 2486 amino acids). The structural region comprised 7461 nt (60–7523), encoding four non-structural proteins (nsP1–4; 2486 amino acids). The structural region contained 3735 nt (7568–11303), encoding 1126 amino acids. Moreover, 11 deletions and two insertions were found in the nsP3 region (nt 4517–5485). For example, six and three nucleotides were found in XJ-160, involving 99 nucleotides. All deletions and insertions were found in the nsP3 region (nt 4517–5485). For example, six and three nucleotides were inserted at positions 4517 and 5299, respectively, while 18 bases were deleted at position 5420. Notably, 11% of amino acids in the XJ-160 E2 protein were different from those in AR339. Some positions in E2 have been suggested as determinants of neuroinvasiveness (Tucker et al., 1993; Ubol et al., 1999). XJ-160 conserved all consensus sequence elements (capsid, E1–3, 6K). Similarity searching via the BLAST server confirmed that this is the seventh full-length Sindbis-like virus genome sequence deposited in the current GenBank database. XJ-160 conserved all consensus sequence elements commonly found in alphaviruses.

Compared with Sindbis virus AR339 (strain HRsp), significant divergence was found in the XJ-160 sequence (Table 2). Globally, XJ-160 shared only 82% identical nucleotides and 91.4% amino acids with the AR339 strain. In contrast, YN87448, Ockelbo and S.A. AR86 strains were more closely related to AR339, with fewer than 6% diverged nucleotides and 3% diverged amino acids. Moreover, 11 deletions and two insertions were found in XJ-160, involving 99 nucleotides. All deletions and insertions were found in the nsP3 region (nt 4517–5485). For example, six and three nucleotides were inserted at positions 4517 and 5299, respectively, while 18 bases were deleted at position 5420. Notably, 11% of amino acids in the XJ-160 E2 protein were different from those in AR339. Some positions in E2 have been suggested as determinants of neuroinvasiveness (Tucker et al., 1993; Ubol et al., 1999).
Table 2. Sequence divergence of XJ-160, YN87448, Ockelbo and AR86 viruses from Sindbis virus AR339

Numbers and percentages, the latter in parentheses, of diverged nucleotides (including insertions and deletions) and diverged amino acids are shown. GenBank accession numbers for strains YN87448, Ockelbo (OCK) and S.A. AR86 (AR86) are AF103734, M69205 and U38305, respectively. NCR, non-coding region.

<table>
<thead>
<tr>
<th>Region</th>
<th>Nucleotides</th>
<th>Amino acids</th>
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<tr>
<td></td>
<td>XJ-160</td>
<td>YN87448</td>
</tr>
<tr>
<td>5'NCR</td>
<td>2 (3.8)</td>
<td>2 (3.4)</td>
</tr>
<tr>
<td>nsP1</td>
<td>202 (12.5)</td>
<td>80 (4.9)</td>
</tr>
<tr>
<td>nsP2</td>
<td>437 (18.0)</td>
<td>135 (5.6)</td>
</tr>
<tr>
<td>nsP3</td>
<td>116 (7.0)</td>
<td></td>
</tr>
<tr>
<td>Conserved</td>
<td>192 (21.3)</td>
<td>54 (5.5)</td>
</tr>
<tr>
<td>Nonconserved</td>
<td>270 (40.1)</td>
<td>66 (9.9)</td>
</tr>
<tr>
<td>nsP4</td>
<td>322 (17.3)</td>
<td>116 (6.3)</td>
</tr>
<tr>
<td>Junction NCR</td>
<td>8 (17.8)</td>
<td>1 (2.2)</td>
</tr>
<tr>
<td>Capsid</td>
<td>115 (14.5)</td>
<td>37 (4.7)</td>
</tr>
<tr>
<td>E3</td>
<td>37 (19.2)</td>
<td>19 (9.9)</td>
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<tr>
<td>E2</td>
<td>254 (20.0)</td>
<td>80 (6.3)</td>
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<td>6K</td>
<td>25 (15.1)</td>
<td>9 (5.5)</td>
</tr>
<tr>
<td>E1</td>
<td>218 (16.5)</td>
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</tr>
<tr>
<td>5'NCR</td>
<td>32 (9.9)</td>
<td>15 (4.7)</td>
</tr>
<tr>
<td>Total...</td>
<td>2114 (18.0)</td>
<td>669 (5.4)</td>
</tr>
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</table>

al., 1994; McKnight et al., 1996; Simpson et al., 1996; Dropulic et al., 1997; Dubuisson et al., 1997). We noted that residue 55 in XJ-160 E2, potentially related to neurovirulence, is Gln.

We performed a phylogenetic analysis and generated a distance matrix tree based on the full-length genomic sequences of the seven Sindbis-like viruses currently available in GenBank (Fig. 1A). XJ-160 forms a separate grouping in this tree, suggesting that it might represent another subgroup or subtype of Sindbis-like viruses. However, as most strains that have been completely sequenced belong to the African/European genetic lineage, the relationship of XJ-160 to the Asian/Australian strains is still elusive. With this in mind, we extended the sequence comparison and phylogenetic analysis to include partial genomic sequence data from representative Asian/Australian isolates of Sindbis-like virus.

By sequence comparison, we found that XJ-160 is most closely related to Kyzylagach virus, a Sindbis-like virus isolated in 1969 in Azerbaijan and partially sequenced recently (Weaver et al., 1997). These two viruses shared 98% identity in a 477 nt E1 region, 99% identity in a 378 nt nsP1 region and 97% identity in a 517 nt nsP4 region. Thus they probably represent different isolates of the same strain. It is noteworthy that Azerbaijan is geographically close to Xinjiang, China, where XJ-160 was isolated. In a phylogenetic tree generated from the E1 nucleotide sequence data (Fig. 1B), XJ-160 clusters with Kyzylagach virus. The separation of XJ160/Kyzylagach from other African/European strains was supported by a statistically significant bootstrap confidence probability (100%). Consistent with previous reports (Weaver et al., 1997; Sammels et al., 1999) Whataroa virus, found in New Zealand and Australia, was in a separate branch of the tree. It probably represents a distinct subgroup or sublineage.

To provide additional insight into the relatedness of XJ-160 to other Sindbis-like viruses, we also compared the XJ-160 sequences with the newly available partial E2 and C sequences of the European (Norder et al., 1996) and Australian (Sammels et al., 1999) strains. Generally, in these regions, XJ-160 shared only ~75% nucleotide sequence identity with European (e.g. isolates R33, EgAR338 and Ockelbo) and Australian (e.g. isolate BH2907) strains, suggesting that XJ-160 has diverged significantly from both African/European and Asian/Australian genetic lineages. However, in a maximum-likelihood tree of E2 sequences (Fig. 1C), which clearly separates the two lineages, XJ-160 is closer to the European/African strains, such as HRsp, EgAR338 and Girdwood S.A., than to the Asian/Australian strains, such as BH2907, V620 and MRE16. Notably, XJ-160 does not cluster with any of the four previously defined groups of Australian strains (Sammels et al., 1999). Similar to trees of full-length and E1 sequences (Fig. 1A, B), XJ-160 is in a separate branch, with strong bootstrap support (100%). Considered together with the sequence divergence between XJ-160 and the other African/European strains, we believe that XJ-160 is a rather isolated Sindbis strain more distant from most previously identified isolates. As shown by Rumenapf et al. (1995), Aura virus is a representative of a separate lineage. Phylogenetic trees of E2 sequences generated by parsimony or distance-matrix methods were very similar to the maximum-likelihood tree (data not shown).
Fig. 1. Phylogenetic analysis of Sindbis-like viruses. (A) Distance matrix tree relating XJ-160 to other Sindbis-like viruses. Distances were based on full-length nucleotide sequences and were calculated using the Kimura two-parameter correction. The tree was reconstructed using the neighbour-joining algorithm. The bar represents 10⁻³ substitutions per 100 nt. The Aura sequence was used for rooting. Programs in the GCG package 10.0 (Genetics Computer Group, Madison, Wisconsin, USA) were used. Similar trees were also obtained using parsimony and maximum likelihood methods (data not shown). (B) Parsimony tree of Sindbis-like viruses based on 477 nt E1 sequences. Methods for inference of phylogeny have been described (Wang et al., 1997c). The tree was constructed by the DNAPARS program in the PHYLIP package 3.573c (Department of Genetics, University of Washington, Seattle, USA). (C) Maximum-likelihood tree of 414 nt E2 sequences from 14 strains of Sindbis-like viruses. The tree was produced by the DNAML program in the PHYLIP package 3.573c. Notably, isolates from all four groups (A–D) of the Australian strains (Sammels et al., 1999) were included in the analysis. (D) Distance-matrix tree of 484 nt C sequences from 14 strains of Sindbis-like viruses. The tree was generated by the DNADIST and NEIGHBOR programs. In (B)–(D), the SEQBOOT and CONSENSUS programs were used to perform bootstrap replication and to generate a majority rule consensus tree from 100 replicates. The lengths of internal branches are proportional only to the bootstrap confidence probabilities (numbers shown). The lengths of external branches in (B)–(D) are uninformative. The GenBank accession numbers of the sequences are as follows: Aura, AF126284; Girdwood S.A., U38304; Babanki, U60394; Kyzylagach, U60396; Whataroa, U60398; BH2907 E2, AFO61690; MK6962 E2, AFO61688; MRE16 E2, AFO61687; AS19016 E2, AFO61685; BH2907 C, AFO61217; MK6962 C, AFO61209; MRE16 C, AFO61210; AS19016 C, AFO61213; MRM39 C, AFO61208; V620 C, AFO61232; RRD800 C, AFO61235; EgAR338 C, AFO61206.

Moreover, a distance-matrix tree based on C sequences (Fig. 1D) lent further support to the separation of XJ-160 from other strains. Thus our results consistently demonstrate that XJ-160 and Kyzylagach viruses probably represent a subtype of Sindbis virus that has been undergoing independent evolutionary development.
In summary, we have isolated a new arbovirus, XJ-160, from mosquitoes collected in Xinjiang, China. XJ-160 is the first Chinese isolate of a Sindbis-like virus. The complete genomic sequence revealed that XJ-160 had diverged significantly from the prototype Sindbis virus.

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References


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