Genetic characterization of Bagaza virus (BAGV) isolated in India and evidence of anti-BAGV antibodies in sera collected from encephalitis patients

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During investigations into the outbreak of encephalitis in 1996 in the Kerala state in India, an arbovirus was isolated from a Culex tritaeniorhynchus mosquito pool. It was characterized as a Japanese encephalitis and West Nile virus cross-reactive arbovirus by complement fixation test. A plaque reduction–neutralization test was performed using hyperimmune sera raised against the plaque-purified arbovirus isolate. The sera did not show reactivity with Japanese encephalitis virus and were weakly reactive with West Nile virus. Complete open reading frame sequence analysis characterized the arbovirus as Bagaza virus (BAGV), with 94.80 % nucleotide identity with African BAGV strain DakAr B209. Sera collected from the encephalitic patients during the acute phase of illness showed 15 % (8/53) positivity for anti-BAGV neutralizing antibodies. This is the first report of the isolation of BAGV from India. The presence of anti-BAGV neutralizing antibodies suggests that the human population has been exposed to BAGV.

An outbreak of Japanese encephalitis (JE) was reported from the Allapuzha, Thiruvanthapuram and Kottayam districts of Kerala state, India, during 1996. Only 33 % (50/150) of the sera collected from hospitalized cases were confirmed as JE by immunoglobulin M (IgM) ELISA. Other clinical specimens were not available for further investigations. Entomological investigations during the outbreak were carried out and 184 mosquito pools collected from the affected area were processed for isolation in 2-day-old Swiss mice by the intra-cranial route (Rodrigues et al., 1980; George et al., 1984). One pool from Culex tritaeniorhynchus showed sickness in inoculated mice. Brains from sick mice were harvested and suspended in 10 % bovalbumin phosphate saline. The suspensions were stored at −70 ºC and designated as the arbovirus isolate (96363). The isolate showed cross-reactivity with anti-JE virus (JEV) and anti-West Nile virus (WNV) immune sera in a complement fixation (CF) test (Pavri & Ghosh, 1969; Rodrigues et al., 1980; Damle et al., 1998).

The isolate did not react with immune sera raised against other circulating arboviruses, including Chandipura (Rhabdoviridae), Sindbis (Togaviridae), Chikungunya (Togaviridae), Kyasanur forest disease (Flaviviridae), Batai (Bunyaviridae) and Dengue (Flaviviridae) viruses (Paul et al., 1970; Rodrigues et al., 1980; George et al., 1984).

In this study, we present the genetic characterization of the arbovirus isolate and serological analysis of available sera collected from encephalitis patients during 1996. The Institutional Animal Ethical Committee approved this work and ethical guidelines were strictly followed according to their recommendations. The arbovirus isolate was plaque-purified to rule out the possibility of isolation of both JEV and WNV from the mosquito pool. The mouse brain stock of the arbovirus isolate was passaged twice in porcine stable kidney (PS) cells to amplify the virus. A single plaque was selected from the first PS cell passage and then subjected to two sequential rounds of plaque purification (total of three plaque-to-plaque transfers), followed by amplification in PS cells. The cell culture supernatant from PS cells was clarified by centrifugation at 3220 g for 10 min at 4 ºC, supplemented with 20 % fetal bovine serum (FBS) and the aliquots were stored at −80 ºC and designated as the arbovirus stocks. Generation of the arbovirus virus-specific polyclonal hyperimmune sera,
plaque reduction neutralization test (PRNT) and genetic characterization studies were performed using the PS-amplified arbovirus stocks. Since the CF test characterized the isolate as a JEV and WNV cross-reactive arbovirus, PRNTs were performed to determine the antigenic relationship among these viruses. An in vitro neutralization test was carried out using PS-adapted JEV (strain 733913), WNV (strain 804994) and the arbovirus isolate (strain 96363), as described previously (Bondre et al., 2007). The threefold-diluted hyperimmune sera were mixed with 100 p.f.u. of each virus and the infectivity was determined in PS cells. The serum dilution showing 80% plaque reduction (ND_{80}) was considered as a neutralizing end point. As shown in Table 1, the highest neutralizing activity was observed with homologous sera. In heterologous neutralization between the arbovirus isolate and WNV, both viruses showed cross-reactivity with each other, although this was weaker than the homologous neutralization. The JEV-specific hyperimmune sera did not neutralize the arbovirus isolate, even at a dilution of 1:5.

As the CF test characterized the 96363 isolate as a JEV- and WNV-reactive arbovirus and the heterologous neutralization showed that it had weak reactivity with WNV, we genetically characterized the isolate. A 1050 nt fragment from the NS5 region of the sample was amplified by RT-PCR using flavivirus-specific universal primers that amplify the partial NS5 fragment from a number of flaviviruses (Kuno, 1998). The genomic RNA of plaque-purified arbovirus grown in PS cells was isolated using QIAamp viral RNA kit (Qiagen) according to the manufacturer's protocol. The RT-PCR amplification was carried out as described by Kuno et al. (1998) and the amplified product was sequenced as described previously (Bondre et al., 2007). BLAST analysis showed 99.90% nucleotide identity (PNI) with African Bagaza virus (BAGV) strain DakAr B209, followed by 95 PNI with Israel turkey meningoencephalitis virus (ITMV). RT-PCR amplification and complete genome sequencing of BAGV-India was achieved by using overlapping primers designed by aligning available flavivirus sequences from GenBank with CLUSTAL_X 1.83 software (Thompson et al., 1997). RT-PCR amplification of overlapping genomic fragments was carried out as described previously (Bondre et al., 2007). PCR products were column-purified (QIAquick PCR purification kit; Qiagen) and both strands were sequenced by using a Big Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems) and an automated Sequencer (ABI Prism 310 Genetic Analyzer). A 10281 nt genomic sequence of BAGV-India (GenBank accession no. EU684972) coding a 3426 aa complete open reading frame (ORF) was obtained. Multiple alignments of nucleotide sequences were carried out by using CLUSTAL_X 1.83. The phylogenetic analysis of the complete genome sequence of BAGV-India was assessed by using MEGA (Tamura et al., 2007). For analysis in MEGA, Jukes–Cantor and nucleotide maximum composite likelihood models were utilized, employing the neighbour-joining algorithm. The topologies generated in the neighbour-joining algorithm were confirmed by using the maximum-likelihood method, as implemented in the software Treefinder 2008, with the gamma-distributed rate variation with four rate categories (HKY +\gamma 4) model of nucleotide substitution (Jobb et al., 2004). The reliability of different phylogenetic groupings was evaluated by using the bootstrap test (1000 bootstrap replications). The genetic distance between different viruses was obtained by using the P-distance model in MEGA. Phylogenetic trees were constructed by using the complete genomic sequence of the Indian BAGV isolate (this study) and complete genomic sequences (from GenBank) of representative strains from different genomic groups in the Flaviviridae. Similarly, phylogenetic analysis of genomic fragments encoding different proteins – nucleocapsid, pre-membrane and membrane, envelope and non-structural (NS) proteins 1–5 – was carried out to understand the relationship between African and Indian BAGV isolates and other flaviviruses.

Comparative analysis of both the Indian and African (AY632545) BAGV complete ORF coding nucleotide sequences showed 94.8% (PNI). The difference of 515 nt (5.2%) resulted in 77 aa (2.24%) differences throughout the ORF of Indian and African (DakAr B209) BAGV isolates (Kuno & Chang, 2007). A difference of 20 aa was documented in the structural protein coding region (14 nt in the nucleocapsid with 2 aa differences, 40 nt in the membrane with 13 aa and 73 nt in the envelope with 5 aa), while a difference of 57 aa was documented in the NS protein coding region (71 nt in the NS1 region with 8 aa differences, 50 nt in the NS2 region with 7 aa, 95 nt in the NS3 region with 9 aa, 48 nt in the NS4 region with 19 aa and 119 nt in the NS5 region with 14 aa). Additionally, compared with BAGV-DakAr B209, one deletion (at nt 7424) and four additions (nt 7438–7439, 7444 and 7463) were documented in the NS4B region of BAGV-India.

Table 1. Homologous and heterologous cross-neutralization test using hyperimmune sera against JEV, WNV and arbovirus (BAGV) isolates

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Hyperimmune sera against:</th>
</tr>
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<tbody>
<tr>
<td>JEV (733913)</td>
<td>WNV (804994)</td>
</tr>
<tr>
<td>JEV</td>
<td>501</td>
</tr>
<tr>
<td>WNV</td>
<td>&lt;5</td>
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<tr>
<td>BAGV</td>
<td>&lt;5</td>
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Phylogenetic analysis using the complete sequence of the Indian BAGV ORF showed that this sequence had a close genetic relationship with the African BAGV-DakAr B209 strain and clustered together with the Culex mosquito-transmitted clade on the phylogram (Fig. 1). Similar tree topologies were obtained with both models (Jukes–Cantor and maximum composite likelihood) that were used to
construct the complete ORF sequence based on the phylogenetic tree obtained by using the neighbour-joining algorithm. The phylogenetic analysis of individual gene sequences coding for nucleocapsid, membrane, NS1, NS2, NS3 and NS4 showed similar tree topologies, which were comparable with complete genome sequence-based analysis (Supplementary Fig. S1, available in JGV Online). The PNI using nucleocapsid and membrane coding gene sequences of Indian and African BAGV isolates was 96.00±1.25 and 92.30±1.20, respectively. Analysis of the NS proteins NS1, NS2, NS3 and NS4 of both the BAGV isolates showed 94.40±0.70, 95.10±0.60, 95.20±0.50 and 95.80±0.60 PNI, respectively. A number of previous phylogenetic studies on flaviviruses mostly attempted to use envelope coding sequences. We also determined the genetic relationship of BAGV-India using the additional envelope sequences of representative members from different Flaviviridae groups. In envelope sequence-based analysis, BAGV-India grouped together with the African DakAr B209 strain (95.90±0.80 PNI) along with other members of the Ntaya virus group of the Flaviviridae (Fig. 2). Envelope sequence analysis of the African BAGV strain (AF372407; Gaunt et al., 2001) showed that it had a closer relationship (99.00±0.40 PNI) with DakAr B209 strain than BAGV-India (94.80±1.70 PNI). Among other members of the Ntaya virus group, ITMV showed a close relationship (93.40–95.50 PNI) with all three BAGV strains, followed by a more distant relationship with Ntaya virus (76.00–77.00 PNI) and Tembusu virus (74.00–75.00 PNI). As partial NS5 sequences from additional

Fig. 1. Phylogenetic analysis of the BAGV complete ORF sequence using the nucleotide maximum composite likelihood model of the neighbour-joining algorithm. Cell fusing agent virus was used as an outgroup in phylogenetic analysis. GenBank accession numbers are given on the figure. Numbers at the nodes indicate bootstrap support for each node. Bar, nt substitutions per site.
Fig. 2. Phylogenetic analysis of BAGV based on partial envelope sequences. The tree was constructed by using MEGA, by the neighbour-joining with nucleotide maximum composite likelihood model. Bootstrap confidence level (1000 replicates) and a confidence probability value based on the standard error test were calculated using MEGA and are indicated at the nodes. Partial envelope sequences of additional viruses (where complete genome sequences were not available) were used in the phylogenetic analysis. Cell fusing agent virus was used as an outgroup in phylogenetic analysis. GenBank accession numbers are given on the figure. Numbers at the nodes indicate bootstrap support for each node. Bar, nt substitutions per site.
members of the *Flaviviridae* were available in GenBank, we performed separate analysis to determine the genetic relationship of BAGV with these viruses (data not shown). With NS5 analysis, both the BAGV sequences grouped together, with 99.90 ± 0.10 PNI, in the Ntaya virus group. However, in NS5 sequence analysis, the nucleotide identities of BAGV and other members of the Ntaya virus group were comparable with envelope sequence analysis. BAGV DakAr B209 and Indian strains showed 95.20–95.30 PNI with ITMV, 76.50–76.60 PNI with Ntaya virus and 75.10–75.30 PNI with Tembusu virus.

We documented one nucleotide insertion and four nucleotide deletions in the complete ORF sequence of Indian and African BAGV strains. The envelope sequence analysis of an additional BAGV strain from Africa indicates a closer genetic relationship with BAGV DakAr B209 than the Indian BAGV strain. These data indicate independent circulation of both the African and Indian isolates in different geographical areas. Although the time and mode of introduction of BAGV in India is unknown, we hypothesize that it may represent a genetic variant of the BAGV strain which originated in the African continent and was dispersed and established in areas with similar climatic conditions and favouring vector multiplication. Dispersal of the flaviviruses from the Old World to the New World and the co-existence of related viruses sharing antigenic, host and vector similarities have been supported by molecular phylogenetic analyses (Sabin, 1959; Gaunt et al., 2001; Chevalier et al., 2004; Mackenzie et al., 2004; Petersen & Marfin, 2005; Gould et al., 2006). However, to determine the precise genetic relationship, geographical origin and epidemiology, full genome sequence data of more strains will be helpful.

We isolated BAGV from a mosquito pool collected during a JE outbreak and studied its genetic relationship with other *Flaviviridae*. Since it was characterized as a JEV and WNV cross-reactive arbovirus (CF test), we determined the antigenic relationship with JEV and WNV by PRNT. Although the heterologous neutralization differentiated these as three distinct arboviruses, we documented weak cross-reactivity between WNV and BAGV (Table 1). The genetic relatedness of BAGV and WNV in several genomic regions might be the reason for antigenic cross-reactivity between these viruses (Kuno & Chang, 2007). We determined the previous exposure of hospitalized encephalitis patients with BAGV by analysing the sera stored at −80°C for anti-BAGV neutralizing antibodies. The neutralization assay was performed with PS cell-adapted BAGV pools, as described previously (Bondre et al., 2007; Sapkal et al., 2007). Only 15% (8/53) of available sera showed reactivity with BAGV, while 24.14% (14/53) were reactive with JEV (733913). Both the anti-JEV and anti-BAGV neutralizing antibody titres (ND80) were in the range of 50–1250. All of the BAGV reactive sera were negative for JEV by IgM ELISA.

Recently, BAGV has been identified as one of the emerging and re-emerging human pathogens that causes febrile illness in humans (Woolhouse et al., 2006). It belongs to the Ntaya group of *Flaviviridae* and has been isolated in the Central African Republic, Cameroon and Senegal, where it circulates between ornithophagic mosquitoes and birds (Digoutte, 1978; Traore-Lamizana et al., 1994; Diallo et al., 2005). It is genetically related to ITMV, which is a serious avian pathogen in the Middle East and southern Africa (Digoutte, 1978; Kuno et al., 1998). The phylogenetic studies using envelope and NS5 sequences clearly suggest that there is a close genetic relationship between ITMV and BAGV. Other members of the Ntaya virus group are genetically distinct from BAGV and ITMV. Our preliminary findings on sera collected during the acute phase of illness from hospitalized patients indicates the presence of anti-BAGV neutralizing antibodies. This suggests that BAGV might be circulating in the area between ornithophagic mosquitoes and birds and incidentally the human population might be exposed to it. These observations need to be strengthened by investigating additional human clinical specimens from the region. However, our preliminary observations need to be confirmed by systematic study of the human population from the Allapuzha, Thiruvanthapuram and Kottayam districts of Kerala to understand the association of BAGV with human infections.

In conclusion, this study indicates the necessity of serious efforts to investigate the likely involvement of BAGV in sporadic human infections and outbreaks in other vertebrates occurring in the region. This can be achieved by developing BAGV-specific serological and molecular diagnostics for testing of human clinical specimens collected from the region. Additional studies addressing the potential of various mosquito species as vectors and birds as amplifying hosts, and sero-surveillance in domestic animals and the human population will add to our understanding of the epidemiology of arboviral diseases.

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**References**


