Isolation and characterization of Bluetongue virus from *Culicoides brevitarsis* (Diptera: Ceratopogonidae) in Okinawa

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**Abstract:** To estimate the vector species of arboviruses affecting cattle, *Culicoides* biting midges were collected by light traps in Naha and Ishigaki cities in Okinawa from 2001–2004. Four and 14 *Culicoides* species were captured in Naha and Ishigaki cities, respectively. One strain of Bluetongue virus (BTV) was isolated from *Culicoides brevitarsis* Kieffer collected in Naha city on December 2003. Genetic and phylogenetic analyses showed that the strain was closely related to the Australian and Asian BTV isolates. Our data indicate that *C. brevitarsis* is likely to be a vector species of BTV in Okinawa.

Key words: *Culicoides*, biting midges, Ceratopogonidae, arbovirus, Bluetongue, Okinawa

**INTRODUCTION**

The genus *Culicoides* of the family Ceratopogonidae of Diptera includes many bloodsuckers, and some of them can transmit pathogens of public and veterinary importance, such as arboviruses, protozoans and filarial nematodes. In southern Japan, outbreaks of several arbovirus diseases of ruminants have been reported (Tsuda, 2000). Most of the viruses, such as the Akabane, Aino, Chuzan and Ibaraki viruses, are probably transmitted by *Culicoides oxystoma* Kieffer (Yanase et al., 2005). As Okinawa belongs to the subtropical zone, its *Culicoides* fauna are somewhat different from those of the mainland of Japan (Hoshino, 1985; Henna et al., 1991; Wada et al., 1996; Sasaki et al., 2004). Notably, *C. brevitarsis* Kieffer, which is a principal vector of ruminant arboviruses in Australia (Muller, 1995), is widely distributed in this region (Henna et al., 1991), but is merely present in the mainland of Japan (Wada, 1999).

Bluetongue (BT) is characterized by congestion, edema and hemorrhage in ruminants and is caused by bluetongue virus (BTV), which is a species of the genus *Orbivirus* within the family Reoviridae and is transmitted by *Culicoides* biting midges. Circulation of BTV has been identified in tropical, subtropical and temperate regions of the world. Usually, sheep show severe clinical symptoms, but
Cattle and goats are asymptomatic. The incursion of BTV was first recognized in Japan in 1974 by sero-surveillance (Miura et al., 1982), and several ovine and bovine BT outbreaks were reported in the 1990s and 2000s (Goto et al., 2004). Several Culicoides species, such as C. imocola Kieffer in Africa, C. sonorensis Wirth & Jones in North America and C. brevitarsis in Australia play important roles in BTV transmission (Mellor et al., 2000). However, the vector species of BTV in Japan remains unclear.

This report describes the isolation of viruses from Culicoides biting midges collected from September 2001 to March 2004 in Naha and Ishigaki cities in Okinawa. The aim of this report is to define candidates for the vector species of arboviruses in Okinawa.

Materials and Methods

Insect collection: The midge collections were conducted at two sites in Okinawa every month from September 2001 through March 2004. One of the sites was a cowshed in Naha city (26°12’ N, 127°41’ E), and the other was a cowshed in Ishigaki city (24°25’ N, 124°9’ E). The collection sites are separated from each other by about 400 km. The collection site in Naha city is within the residential area and that in Ishigaki city is surrounded by pasture and woods. Midge were collected once a month by using light traps that were equipped with a 6-W black light tube and a down-draft suction fan. The traps were operated overnight. The live midges were sent to the laboratory in Kyushu Research Station of the National Institute of Animal Health and were kept for 10 or 2 days on 10% sucrose at 25°C. Throughout the incubation period, the midges digested blood meals. After completion of the incubation period, the survivors were sorted into species based on the morphological keys of Kitaoka (1984a, b) and were stored at −80°C until virus isolation. Midges were pooled on the basis of collection date and species.

Virus isolation: The collected midges were processed as described previously (Yanase et al., 2005). Briefly, each pool of midges excluding males was homogenized in culture medium consisting of Eagle’s minimum essential medium and 0.295% tryptose phosphate broth supplemented with 10 µg/ml gentamicin sulfate and 2.5 µg/ml amphotericin B. The homogenized suspension was centrifuged at 860 g for 10 min, and the supernatant was inoculated onto established cell lines (BHK-21 and HmLu-1). Inoculated cells were incubated with culture medium in a glass tube for 7 days with gentle rotation. All cultures were pooled and passaged twice in the same manner if no cytopathic effect was observed.

Virus characterization: Isolated viruses were identified by dot immunobinding assays with antibodies against the Akabane, Aino, Chuzan, Ibaraki and Bluetongue viruses (Yoshida and Tsuda, 1998). Viral RNA was extracted from the supernatant of infected cells using the High Pure Viral RNA Kit (Roche, Basel, Switzerland) according to the manufacturer’s instructions. To amplify the partial sequences of segments 3 and 10 of BTV, RT-PCR was carried out with the Titan™ One Tube RT-PCR Kit (Roche) and BTV specific primers: A196 (5’ accgcacacgccttataagtgtagttag 3’) and A203 (5’ atacgctgcctccgagtccttacc 3’) for RNA segment 3, and B 4 9 (5’ gttaaaaagtgctgctgtcagccttgcttg 3’) and B50 (5’ gtaagtgtatatagcgcgca 3’) for RNA segment 10 (Pritchard et al., 2004). The PCR products were purified using the PCR purification kit (Qiagen, Valencia, CA) and were subjected to automated sequencing with each BTV-specific primer described above. The obtained data were compared with the published BTV sequences using the Basic Local Alignment Search Tool (BLAST) (National Center for Biotechnology Information). The sequence data were deposited in SAKURA of the DNA Data Bank of Japan (DDBJ) under accession
numbers AB504735 (for segment 3) and AB504736 (for segment 10). The sequence data was aligned with other BTV sequence data obtained from GenBank database with CLUSTAL W (Thompson et al., 1994) and the evolutionary history was inferred using the Neighbor-Joining (NJ) method (Saitou and Nei, 1987). Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

R: HJAIH 6C9
D: >H8JHH>DC

A total of 4,298 and 4,499 midges were captured in Naha and Ishigaki cities, respectively, throughout the research period (Table 1). The Culicoides biting midges collected in Naha city consisted of four species, and most were identified as C. brevitarsis (93.3%). Fourteen Culicoides species were collected in Ishigaki city, and the most abundant species was C. arakawae Arakawa (68.3%). The two nextmost abundant species in Ishigaki city were C. oxystoma (14.7%) and C. brevitarsis (6.7%). The difference in species composition between the two sites was related to the environments around the cowsheds. The collection site in Naha city was located within the city area, whereas that in Ishigaki city was surrounded by a natural area, where many breeding habitats might be available for various Culicoides species. (Table 1)

A total of 151 pools of Culicoides biting midges were tested for the purpose of virus isolation. A strain of BTV was isolated from C. brevitarsis collected on December 16, 2003 in Naha city and was designated as ON-2/C/03. No other virus isolation was achieved in this study. The partial sequence of segments 3 and 10 of ON-2/C/03 was determined. A BLAST search showed that segment 3 of ON-2/C/03 was genetically closest to the Kinmen (KM) isolate from a goat in Taiwan (98.6% in nucleotide identity) (Ting et al., 2005). Segment 10 of ON-2/C/03 is closely related with the Australian BTV isolates V4014 and V3692 (96.2% in nucleotide identity) (Pritchard et al., 2004). Phylogenetic tree analysis of the segment 3

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of Captured midges*</th>
<th>No. of pools tested**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Naha</td>
<td>Ishigaki</td>
</tr>
<tr>
<td>C. arakawae</td>
<td>3 (0)</td>
<td>3075 (125)</td>
</tr>
<tr>
<td>C. oxystoma</td>
<td>286 (30)</td>
<td>663 (185)</td>
</tr>
<tr>
<td>C. brevitarsis</td>
<td>4005 (226)</td>
<td>303 (56)</td>
</tr>
<tr>
<td>C. peregrinus</td>
<td>183 (22)</td>
<td></td>
</tr>
<tr>
<td>C. dubius</td>
<td>72 (4)</td>
<td></td>
</tr>
<tr>
<td>C. brevipalpis</td>
<td>42 (4)</td>
<td></td>
</tr>
<tr>
<td>C. verbosus</td>
<td>33 (9)</td>
<td></td>
</tr>
<tr>
<td>C. actoni</td>
<td>20 (0)</td>
<td></td>
</tr>
<tr>
<td>C. sumatrae</td>
<td>17 (0)</td>
<td></td>
</tr>
<tr>
<td>C. wadaei</td>
<td>15 (2)</td>
<td></td>
</tr>
<tr>
<td>C. nipponensis</td>
<td>8 (0)</td>
<td></td>
</tr>
<tr>
<td>C. jacobsoni</td>
<td>6 (0)</td>
<td></td>
</tr>
<tr>
<td>C. longiensis</td>
<td>1 (0)</td>
<td></td>
</tr>
<tr>
<td>C. flavipunctatus</td>
<td>1 (0)</td>
<td></td>
</tr>
<tr>
<td>C. morisitai</td>
<td>4 (0)</td>
<td></td>
</tr>
<tr>
<td>Culicoides spp.</td>
<td>60 (5)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4,298 (256)</td>
<td>4,499 (412)</td>
</tr>
</tbody>
</table>

* Number of captured males is given in the parenthesis.
** Pools including midges captured in Naha city in the parenthesis.

RESULTS AND DISCUSSION

A total of 4,298 and 4,499 midges were captured in Naha and Ishigaki cities, respectively, throughout the research period (Table 1). The Culicoides biting midges collected in Naha city consisted of four species, and most were identified as C. brevitarsis (93.3%). Fourteen Culicoides species were collected in Ishigaki city, and the most abundant species was C. arakawae Arakawa (68.3%). The two nextmost abundant species in Ishigaki city were C. oxystoma (14.7%) and C. brevitarsis (6.7%). The difference in species composition between the two sites was related to the environments around the cowsheds. The collection site in Naha city was located within the city area, whereas that in Ishigaki city was surrounded by a natural area, where many breeding habitats might be available for various Culicoides species. (Table 1)
showed that ON-2/C/03 clustered with Asian and Australian isolates, but was far from American and European isolates (Fig. 1). The segment 3 sequence has been used to determine the topotype of BTV (Pritchard et al., 2004). Also, the genetic variation of the segment 10 sequence is probably related to the geographical origin of BTV (Pritchard et al., 2004). Although sequencing of the other segments and serotyping would be necessary, these results indicated that ON-2/C/03 was evolved from a common gene pool in Asia and Australia. (Fig. 1)

The BTV isolation from C. brevitaris suggested that this species is an important vector in Okinawa, as in Australia (Muller, 1995). Former reports touched on BTV isolation from C. brevitaris in Okinawa (Takayoshi et al., 1994; Goto et al., 2004), but no detailed information was available until the present study. Although incursion of BTV has been sporadically detected, no clinical case of BT has been observed in Okinawa so far. However, BTV infection has occasionally induced severe developmental brain defects in calves (Wouda et al., 2008), suggesting the necessity of surveillance for BTV in Okinawa.

At both collection sites, C. brevitaris was captured by light traps all year round. The winter in Okinawa is sufficiently warm to support midge activity, indicating that there is no vector-free period. C. brevitaris is distributed in most of the Okinawa Islands (Henna, et al., 1991), and its larvae breed in bovine dung in the pasture (Cannon and Reye, 1966), whereas larvae of C. oxystoma breed in wet soil, such as the surfaces of paddy rice fields (Kitaoka and Morii, 1963). There are some difference in vector species, activity periods and breeding sites between Okinawa and other parts of Japan; therefore particular protocols to control and prevent arbovirus infections should be prepared for the livestock in Okinawa. Recently, an obvious linkage between Okinawa and East Asian countries was observed in several epidemics of bovine ephemeral fever (Aizawa et al., 2008). Because Okinawa has been one of the entrance sites of arbovirus incursion from overseas, continuous virological and entomological surveillance should be conducted to assess the probable risk of arbovirus diseases.

It is certain that global worming affects
the distribution of *Culicoides* species. Recently, it was shown that *C. imicola* is more widely distributed than had been previously recorded (Wilson and Mellor, 2008). It is possible that *C. breviratis* has already expanded its distribution into the mainland of Japan. The current distribution of this species should be clarified in regions other than Okinawa.

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**REFERENCES**


