ENCEPHALITIS AND RETARDED GROWTH OF CHICKS CAUSED BY SITIAWAN VIRUS, A NEW ISOLATE BELONGING TO THE GENUS FLAVIVIRUS

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Abstract. A new virus named Sitiawan virus (SV) was isolated from sick broiler chicks in chicken embryos. The virus replicated well with cytopathogenic effect (CPE) in the chicken B-lymphocyte cell line LSCC-BK3. The virus was an enveloped RNA virus of approximately 41 nm in size with hemagglutinating activity (HA) to goose erythrocytes. It was cross-reactive with Japanese encephalitis virus (JEV), a member of flaviviruses by HA inhibition tests but not by cross-virus neutralization tests. The cDNA fragment of NS5 gene was amplified with primers corresponding to NS5 gene of flaviviruses. The nucleotide sequences were 92% homologous to Tembusu virus, a member of the mosquito-borne virus cluster of the genus Flavivirus. In cross-neutralization tests with Tembusu virus, antisera to SV did not neutralize Tembusu virus, and antiserum to Tembusu virus neutralized more weakly to SV than against homologous virus. These results indicate that SV is a new virus which can be differentiated serologically from Tembusu virus but is otherwise similar with respect to nucleotide sequence. The virus causes encephalitis, growth retardation, and increased blood glucose levels in inoculated chicks.

INTRODUCTION

Infectious-stunting syndrome, spiking-mortality syndrome (SMS), and other diseases of unknown etiology have often caused the fall of productivity in chicken broiler farms worldwide. These syndromes can be confusing, especially in tropical countries where infectious agents such as arboviruses are considered the causative agents in addition to other pathogens. Many arboviruses including alphaviruses and flaviviruses have been studied extensively as causative agents of hemorrhagic fever or encephalitis in humans and other mammals. In contrast, only four chicken arboviruses have been identified as causes of disease in domestic poultry and farm-reared game birds. These arboviruses are the Eastern equine encephalitis virus, Western equine encephalitis virus, Highland J virus and Israel turkey meningoencephalitis virus. This paper describes the isolation of a new flavivirus from chicks and demonstrates its pathogenicity in these poultry.

MATERIALS AND METHODS

Field samples. In a broiler farm in the Sitiawan district of Perak state, Malaysia, an outbreak of a disease characterized by stretching of legs or impairment of mobility was observed in 4–6 week-old chicks. Five 4-week-old chicks showing such signs were used for etiological studies. Since the signs were similar to REO virus infection, a 10% homogenate of the pooled digital flexor tendons was filtered through 450 nm filters and used as an inoculum for virus isolation.

Chicken sera used for antibody assays. Serum samples were collected from 3 farms in the Sitiawan district. Ten to 38 sera were collected at each monitoring period. Each farm was monitored 2–6 times (Table 3).

Virus isolation in specific pathogen-free embryonating chicken eggs. The filtered sample of the pooled digital-flexor tendons was inoculated into yolksacs of 6- or 7-day-old specific-pathogen free embryonating chicken eggs (ECE) at 0.1 ml per egg. Embryos that died 32–120 hr after inoculation were pooled, homogenized, and centrifuged. The supernatants were inoculated into another set of ECE. The virus was named Sitiawan virus (SV) and the serially propagated virus in ECE was named SV-ECE.

Isolation of BK3-cell adapted SV. The LSCC-BK3 cells (BK3 cells: transformed chicken B lymphocytes by avian leukosis virus, ALV) were grown in Ham's F10 medium containing 10% tryptose-phosphate broth and 10% fetal calf serum (GM). One-tenth mL of BK3 cell suspension (1 × 10⁶ cells/mL) was mixed in a well of a 24-well plastic plate with an equal volume of SV at the sixth passage level in ECE (SV-ECE) and kept in a CO₂ incubator at 37°C for 1 hr. After incubation, 0.8 mL GM was added and incubation continued for 3 days. Two further passages in BK3 cell suspension using culture fluids from the previous passage were carried out. An apparent inhibition of cell replication or CPE was considered positive for virus replication. The virus adapted to BK3 cells was named SV-BK3.

Titration of SV-BK3. The titration was carried out in BK3 cell cultures using 96-well plastic plates. Ten-fold serial dilutions of viral samples (0.1 mL) were inoculated into 0.1 mL BK3 cell suspension (1 × 10⁶ cells/mL) and incubated for 4 days. Three or 4 wells were used for each dilution. The cultures that showed apparent inhibition of cell replication were considered virus positive, and the 50% tissue culture infective dose (TCID₅₀) was calculated by Reed and Muench's method.

Virus replication in other cell cultures. Besides BK3 cells, cloned porcine kidney cells (CPK), MA104 cells (MARC 145), African green monkey kidney cells (VERO), and chicken embryo fibroblast cultures were used for virus replication. These cells were infected with SV-BK3 by routine procedures.

Virus titration of experimentally infected chicken tissues. One percent or 10% tissue homogenates were made with GM and centrifuged at 8,000 rpm for 15 min using a microcentrifuge (Eppendorf 5417R). Serial 10-fold dilutions of supernatant were inoculated into BK3 cell cultures.

Physicochemical properties of the isolates. SV-BK3 (fifth passage level) was used. Ether sensitivity, filterability,
and replication in the presence of 5-iodo-deoxyuridine were tested as described previously.6,7

Hemagglutination and hemagglutination inhibition tests. The hemagglutinin of SV was prepared by the sucrose-acetone extraction method from the brain of a mouse showing neurologic signs after inoculation intracebrally with 0.03 mL of SV-BK3.8 The hemagglutinin of Japanese encephalitis virus (JEV) was obtained from a commercial diagnostic kit for JEV (Kyoto Biken, Japan). The hemagglutination (HA) test was carried out using goose erythrocytes as described.9 The HA inhibition test (HI) was performed at 4°C for 18 hr using antisera (described in following section) treated with acetone and absorbed with goose erythrocytes.9

Serum neutralization test. Viruses were VERO-cell adapted SV (fourth passage), Tembusu virus (obtained through the courtesy of Dr. E. A. Gould, Institute of Virology and Environmental Microbiology, Oxford, United Kingdom) grown serially 4 times in VERO cells and JEV (Jagar 01 strain, obtained through the courtesy of Dr. O. Ito, National Veterinary Assay Laboratory, Tokyo, Japan). Antisera against SV and Tembusu virus were collected from chicks 25 days after intraperitoneal inoculation with 10⁶ and 10⁷ TCID₅₀ of SV and Tembusu virus, respectively. The antisemum to JEV was from a pig infected with JEV, strain AS-6 (a gift from Dr. Y. Goto, National Institute of Animal Health). Sera collected from experimentally infected chicks 1–21 days post-inoculation were also used. Serial two-fold dilutions of heat-inactivated sera were mixed with 200 TCID₅₀ of virus, incubated at 37°C for 1 hr and then inoculated into VERO cells.9 Results were read when definite CPE was observed in control cultures. The serum neutralizing (SN) antibody titer was expressed as the reciprocal of the serum dilution that inhibited CPE.

Indirect fluorescent antibody test. The conventional indirect fluorescent antibody test (IFA) method was used to detect antibody to SV in chicks in farms. Infected BK3 were washed twice with phosphate buffered saline (PBS) and suspended in PBS. Ten μl of the suspension was dispensed and then aspirated immediately in a hole of a 21-hole FA slide (Cell-Line Associates, Inc., USA) for the cells to adhere. The cells were fixed with cold acetone, reacted with chicken serum diluted 1/100, washed thoroughly with PBS, and stained with anti-chicken IgG goat serum labeled with FITC (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

Reverse transcription polymerase chain reaction. The viral RNA was extracted from infected cell culture fluids using a RNA extraction reagent (TRIzol, GibcoBRL, Grand island, NY) following the manufacturer’s recommendations.

A commercial kit (Takara RNA PCR kit, Takara Biomedicals, Otsu, Japan) was used for the reverse transcription-polymerase chain reaction (RT-PCR). For cDNA synthesis a mixture of viral RNA, random 9 mers, and enzyme mixture (total 20 μl) was incubated at 42°C for 90 min and then at 99°C for 5 min. Temperature cycles used were one cycle of heat denaturation at 94°C for 5 min, 35 cycles of 3 temperature changes (94°C for 1 min, 53°C for 1 min, and 72°C for 1 min) for amplification and one cycle for final extension at 72°C for 10 min.

Primers. Primers (Nakarai Chemicals, Inc., Tsukuba, Japan) used for amplifying and sequencing the genomic regions (about 1 kb long) at the 3’ terminus of the NS5 gene of flaviviruses are shown in Table 1,10 as well as newly designed primers for the same purpose.

Nucleotide sequencing. This was performed as described using the dye deoxy terminator kit (Applied Biosystems, Foster City, CA).10 PCR products were directly sequenced with the use of the ABI model 310 genetic analyzer (Perkin Elmer, Norwalk, CT). Nucleotide sequences were edited and compiled using the Sequence Navigator computer program (version 1.0; Applied Biosystems, Norwalk, CT).

Phylogenetic analysis. Phyllograms for the entire sequence (about 1 kb between primers FU1 and cFD3) were obtained by the use of the UPGMA method (GENETYX Co. Ltd., Tokyo, Japan). Corresponding sequence data of reference viruses were obtained from GenBank (accession no.: Bagaza virus, AF013363; Israel turkey meningoencephalitis virus, AF013377; Nyata virus, AF013392; Tembusu virus, AF013408; and ThCar virus: AF03409).

Experimental inoculation of chicks. The chicken experiment was performed following the guidelines of The Veterinary Research Institute, Ipoh, Malaysia, for the use of experimental animals. Three chick-inoculation experiments with SV were performed using one-day-old specific pathogen-free chicks to test the pathogenicity of SV. Each group was kept in a bio-isolator. The room lights were kept lit the entire day with feed and water freely available. In experiments 1 and 2, each of 24 and 26 chicks was divided into 3 groups. Each was inoculated with 0.5 mL of SV-ECE at the sixth passage level in ECE (10⁶ EID₅₀/mL), SV-BK3 at the sixth passage level in BK3 cultures (10⁶ EID₅₀/mL), or 0.5 mL PBS. The number of chicks in each group is shown in Table 2. Clinical signs were observed daily, and blood glucose levels were tested once every 2–5 days. Body weights were taken once every 4–7 days and histopathology when the chicks were sacrificed (Table 2). In experiment 3, 10 chicks were inoculated with SV-BK3 as described above and

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**Table 1**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’–3’)</th>
<th>Genomic position*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1 (F)</td>
<td>AAGCTGACACCCACCCCTTTGGAC</td>
<td>8666</td>
<td>Newly designed</td>
</tr>
<tr>
<td>cFD2</td>
<td>GTGTCGCCAGCGCGGCTCAG</td>
<td>9255</td>
<td>Kuno and others</td>
</tr>
<tr>
<td>FU1 (F)</td>
<td>TACCAACATGTTGGAAGAGAGAA</td>
<td>8993</td>
<td>ditto</td>
</tr>
<tr>
<td>cFD4</td>
<td>ACCACACCATCATCTCCGCT</td>
<td>9648</td>
<td>ditto</td>
</tr>
<tr>
<td>12 (F)</td>
<td>TCCCGAGAGGACGGAGGCTCAA</td>
<td>9530</td>
<td>Newly designed</td>
</tr>
<tr>
<td>cFD3</td>
<td>AAGCATGTCTTCCCGTGTACCCCA</td>
<td>10077</td>
<td>Kuno and others</td>
</tr>
</tbody>
</table>

* Numbers are genomic positions of yellow fever virus (GenBank accession no. K02749). (F) = forward primer; (R) = reverse primer. The primers are universal for most flaviviruses, except B1 (F) and 12 (F), which is specific for Sitiawan virus.
Clinical signs, blood glucose levels, body weights, and histopathological changes shown in 1-day-old chicks inoculated with Sitiawan virus (SV)*

<table>
<thead>
<tr>
<th>Exp no.</th>
<th>Group</th>
<th>No. of chicks</th>
<th>Inoculum</th>
<th>Clinical signs†</th>
<th>Blood glucose levels§</th>
<th>Body weight¶</th>
<th>Histopathology¶</th>
<th>Brain‡</th>
<th>Pancreas</th>
<th>§</th>
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<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>8</td>
<td>SV-ECE</td>
<td>Torticollis and imbalance (recovered)</td>
<td>6/8</td>
<td>141.25±6.88 (130–170)</td>
<td>8/8</td>
<td>8/8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>8</td>
<td>SV-BK3</td>
<td>Tremors and depression (sacrificed)</td>
<td>7/8</td>
<td>136.25±9.40 (85–160)</td>
<td>8/8</td>
<td>6/8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>PBS</td>
<td>None</td>
<td>None</td>
<td>0/8</td>
<td>181.25±17.90 (150–210)</td>
<td>0/8</td>
<td>0/8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>8</td>
<td>SV-ECE</td>
<td>Imbalance (recovered)</td>
<td>7/8</td>
<td>116.25±17.56 (90–145)</td>
<td>7/8</td>
<td>8/8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>SV-BKV</td>
<td>None</td>
<td>None</td>
<td>8/9</td>
<td>123.3±18.35 (110–165)</td>
<td>6/7</td>
<td>6/7</td>
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<tr>
<td>6</td>
<td>11</td>
<td>PBS</td>
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<td>None</td>
<td>1/11</td>
<td>150.5±9.94 (130–170)</td>
<td>0/11</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>10</td>
<td>SV-BKV</td>
<td>Circling and torticollis (sacred)</td>
<td>NT</td>
<td>NT</td>
<td>6/8</td>
<td>5/8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* One-day-old SPF chicks were inoculated intraperitoneally with 0.5 ml of Sitiawan virus serially propagated 6 times in embryos (SV-ECE, 10^8 ELD<sub>50</sub>/mL) or Sitiawan virus serially propagated 5 times in BK3 cell cultures (SV-BKV, 10^7 TCID<sub>50</sub>/mL). SV was isolated from chickens that died 7–8 days after inoculation. The farm in which SV was isolated was one of these farms.

† One chick each in groups 1, 2, 4, and 7 showed clinical signs on 9, 11, 9, and 12 days post-inoculation, respectively.

‡ Number of chicks with blood glucose levels higher than 300 mg/dL/Number of chicks tested between 1 and 7 days post-inoculation.

§ The time of post-mortem examination was 21 days post-inoculation in group 1, 23 days post-inoculation in group 5, and 28 days post-inoculation in groups 4 and 6.

¶ Number of chicks with histopathological changes of perivascular cuffing and/or glial nodule formation in the brain and lymphocyte infiltration and/or follicle formation in the pancreas among tested chicks.

NT = not tested.

Exp’s = experiment.

Sacri fi ced sequentially between 1 and 18 days post-inoculation to investigate virus distribution in tissues and development of histopathological changes.

**Blood glucose levels.** In Malaysia, occurrences of SMS—which was characterized by sudden death of chicks with hyperglycemia have been reported in many chicken farms. The farm in which SV was isolated was one of these farms. Therefore, chicks inoculated with SV were tested for blood glucose levels during the experimental period in relation to SMS. This was tested between 3 to 4 PM using a portable blood glucose concentration meter (Glucocard, Hoechst Marion Russel, Tokyo, Japan). The blood glucose levels of infected and control groups were compared by the Student’s t-test. Blood glucose levels of test samples that were higher or lower than the mean ± 2 standard deviations (SD) of the control group were considered abnormally high or low values.

**Histopathology and electron microscopy.** Chicks in experiment 3 were examined to study the sequential development of histopathological changes. One or two chicks with no clinical signs at 1, 3, 4, 6, and 8 days post-inoculation, one showing neurological signs at 12 days post-inoculation, and one with no signs at 18 days post-inoculation were examined histopathologically. Thirty-one infected chicks (groups 1, 2, 4, and 5) and their 19 control chicks (groups 3 and 6) in experiments 1 and 2 were also examined 21–28 days post-inoculation to investigate whether SV-ECE and SV-BK3 cause similar histopathologic changes. The main organs of the chicks were examined microscopically after hematoxylin and eosin staining. For electron microscopy, infected and control BK3 cells fixed with glutaraldehyde were used.

**RESULTS**

**Virus isolation in ECE.** All five embryos inoculated with the pooled tendon specimen died 3–5 days post-inoculation. In subsequent passages in ECE, four embryos were inoculated with a 10% homogenate of the pooled dead embryos. At the sixth passage, the embryo homogenate had an infectivity titer of 10<sup>5</sup> ELD<sub>50</sub>/mL. This homogenate was used for chicken inoculation experiments and the virus was named Sitiawan virus (SV) after the name of the district in which it was isolated.

**Isolation of BK3-cell adapted SV.** After three serial passages in BK3 cells, the virus induced marked CPE in the cultures 3–4 days after inoculation (SV-BK3). The infectivity titer of the culture fluid was ~10<sup>6</sup> TCID<sub>50</sub>/mL.

**Replication of SV-BK3 in various cells.** The virus grew (10<sup>3</sup> TCID<sub>50</sub>/mL) in CEF although the CPE was not prominent. The virus also grew in CPK cells, MARK-145 cells, and VERO cells without apparent CPE. Further subcultures of the virus were carried out in VERO cells. The CPE became apparent after four serial passages in the cultures. The infective titer was ~10<sup>5</sup> TCID<sub>50</sub>/mL.

**Hemagglutination.** The sucrose-acetone extract from the brain of a mouse that died 7–8 days after intracerebral inoculation with SV showed HA activity to goose erythrocytes at pH 6.0–6.6. The highest titer (12,800) was at pH 6.2. No
GUISHABLE FROM ALV (Figure 1A). The virus was clearly distinguished in the endoplasmic reticulum and endoplasmic vacuoles of BK3-infected cells. The virus was detected in the endoplasmic reticulum and endoplasmic vacuoles with a diameter of 40 nm. The infectivity was 10^{3.5} TCID_{50} /mL after the addition of and mixing with an equal volume of diethyl ether. The infectivity was 10^{3.5} TCID_{50} /mL after filtration through a Sartorius membrane filter with a pore size of 50 nm. To define the nucleic acid type of SV, the effect of 10^{-3.5} M 5-iodo-deoxyuridine (IUDR) was tested in CEF cultures. Avian REO virus and duck enteritis virus which were isolated in Malaysia were used as references for RNA and DNA viruses, respectively. The growth of SV, avian REO virus and duck enteritis virus in CEF cultures in the presence and absence of IUDR were 10^{5.5} and 10^{5.5}, 10^{4.9} and 10^{4.9}, and 10^{5.0} and 10^{7.0} TCID_{50}, respectively.

Antigenicity. In cross HI tests between SV and JEV, both SV and JEV showed cross-reactivity with heterologous antibodies, although it was weaker than the homologous reaction. No cross-reaction was observed in the SN test. In cross SN tests between SV and Tembusu virus, antiserum against SV neutralized SV at a serum dilution of 1:1280 but did not neutralize Tembusu virus at a dilution of 1:5. Antiserum against Tembusu virus neutralized homologous virus at a serum dilution 1:160 and SV at 1:20 (Table 3).

Electron microscopy. Spherical and enveloped virus particles with a diameter of 40–50 nm (average 41 nm) were observed in the endoplasmic reticulum and endoplasmic vacuoles of BK3-infected cells. The virus was clearly distinguishable from ALV (Figure 1A–C).

Molecular biologic characterization of SV. Expected sizes of products were generated by PCR with use of 3 pairs of primers. One-thousand three-hundred and forty-one nucleotides near the 3' terminus of the NS5 gene were sequenced (GenBank accession number AB026994). Computer analysis of the 1,030 nucleotides showed identity of 92.1, 89.9, 73.8, 75.7, and 75.7%, with the sequences of corresponding sites of Tembusu, ThCar, Ntay, Israel Turkey Meningoencephalitis, and Bagaza viruses that belong to the genus Flavivirus. Similarly, amino acid homology of SV was 98.8%, 99.1%, 87.8%, 88.0%, and 88.9% with these viruses, respectively. Phylogenetic analysis also showed that SV had a very close genetic relationship with Tembusu and ThCar viruses (Figure 2).

Clinical changes in infected chicks. Chicks inoculated with SV-ECE and SV-BK3 succumbed to the same clinical signs. Although these chicks showed no apparent clinical illnesses until 8 days post-inoculation, 1 chick in experiment 1, 2 in experiment 2, and 1 in experiment 3 developed central nervous signs such as torticollis, circling movement, imbalance, tremors and depression 9–12 days post-inoculation (Table 2). Two recovered 5 and 6 days after the onset, and two were moribund and sacrificed for virologic and histopathologic studies. The control chicks were apparently healthy.

Body weight. Significant (P < 0.01) retarded growth was observed in the infected chicks 1 (experiment 1) and 2 weeks (experiment 2) post-inoculation and thereafter. The average body weights of infected chicken groups significantly decreased to 68% to 82% of those in the respective control group on 19 and 21 days post-inoculation (Figure 3 and Table 2). The average body weight of chicks in group 4 was 74% of the control 28 days post-inoculation. No significant difference was found between the body weight of chicks inoculated with SV-EV and SV-BK3.

Blood glucose levels. The average blood glucose levels (mg/dl) of control chicks were in the range of 243 ± 15.5 to 270 ± 18.4 at 1 and 20 days post-inoculation. Sixty-six to 90% of the chicks in the four infected groups showed higher blood glucose levels (300–600 mg/dl) than the control chicks 1–7 days post-inoculation (Table 2). Sequential changes of blood glucose levels in the infected group with SV-ECE and the controls are shown in Figure 4. No chicks showed a blood glucose level < 150 mg/dL (regarded as hypoglycemic in chicks).

SN antibody in experimentally infected chicks. Serum neutralizing antibody was first detected 8 days post-inoculation (Table 4) and increased to 320 at 21 days post-inoculation.

Distribution of the virus in experimentally inoculated chicks. In experiment 3, four chicks that showed no clinical signs at 1, 4, 6, and 8 days post-inoculation and one that manifested apparent neurological signs at 12 days post-inoculation were sacrificed for virus titration in tissues. One chick in group 5 of experiment 2 that showed severe depression and tremor was also tested at 11 days post-inoculation. In these chicks, viremia was detected in chicks at 1, 4, and 6 days post-inoculation but not 8 days post-inoculation thereafter (Table 4).

The virus was detected in all tissues and fecal samples at high titers in chicks that showed viremia. The titer was apparently high in the brain of a chick tested without clinical signs on 8 days post-inoculation and two chicks with neurological signs.

Histopathological changes. Perivascular cuffing and glial nodule formation in the brain and lymphocyte infiltration and follicle formation in the pancreas were the main changes observed consistently in almost all infected chicks with SV-ECE and SV-BK3 (Table 2 and Figure 1D–E). The perivascular cuffing and lymphocyte infiltration were seen 3 days post-inoculation and the glial nodule formation and lymphocyte follicle formation were observed later (8 days post-inoculation). In chicks that showed neurologic signs, changes with greater severity were observed. No significant changes were found in the bursa of Fabricius, heart, intestine, kidney, liver, lung, spleen, and thymus.

Serologic reactors against SV in the field. Although positive reactors detected by the IFA test were found in high proportion (70%) in 1-week-old chickens, the reactors decreased in numbers in 2–4-week-old chicks as shown in Ta-
Figure 1. A–C. Electron micrographs of BK3 cells 18 hours after infection with Sitiawan virus. Bar: 100 nm. A) Many small spherical virus particles are shown in endothelial reticulum. B) Enveloped viruses are shown in endothelial vacuoles. C) Sitiawan virus and avian lekosis virus (ALV) which is persistently infected in the BK3 cells are shown. The arrowhead indicates Sitiawan virus and the arrow some ALV particles locating in space between cells. D–E. Histopathological changes in chicks infected with Sitiawan virus. Bar: 50 μm. D) Follicle formation and infiltration of lymphocytes in the pancreas. E) Glial nodule in the cerebrum. F) Perivascular cuffing in the cerebellum.
CHICK ENCEPHALITIS CAUSED BY SITIAWAN VIRUS

FIGURE 2. Phylogenetic tree of Ntaya group viruses using nucleotide sequence of NS 5 gene of Sitiawan virus. This was obtained by use of the UPGMA method (GENETYX Co. LTD. Tokyo, Japan). The number in the figure shows estimated evolutionary distance.

Israel turkey meningoencephalitis virus.

FIGURE 4. Changes in blood glucose levels of chicks infected with Sitiawan virus. Groups 5 and 6 in experiment 2 (see Table 2) are shown. The vertical bar indicates standard deviation. \( P < 0.05; P < 0.01 \).

TABLE 4

<table>
<thead>
<tr>
<th>Tissue tested</th>
<th>Day after inoculation</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Serum</td>
<td>4.5†</td>
</tr>
<tr>
<td>Cerebrum</td>
<td>NT</td>
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<td>Cerebellum</td>
<td>NT</td>
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<tr>
<td>Lung</td>
<td>3.8</td>
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<tr>
<td>Liver</td>
<td>2.5</td>
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<td>Spleen</td>
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<td>Kidney</td>
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<td>Pancreas</td>
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<tr>
<td>Thymus</td>
<td>&gt;6.5</td>
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<td>Bursa Fabricius</td>
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<td>Duodenum</td>
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<td>Feces</td>
<td>3.5</td>
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<tr>
<td>SN antibody titer</td>
<td>&lt;2</td>
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</table>

*1-day-old chicks were inoculated intraperitoneally with 0.5 mL of SV-BK3 (10^{7.5} TCID_{50}/mL). Each chick was tested on the day indicated.
† Log 10 TCID_{50}/g.
NT = Not tested.

Common HA antigens were also observed between SV and JEV\(^{14}\). The serologic relationship, however, was not observed between the viruses in the SN tests, indicating that the new isolate belongs to an antigenic complex different from JEV\(^{15,16}\). On the other hand, the comparison of genomic sequences at the 3’ terminus of the NS5 gene of SV with that of viruses of the Ntaya antigenic complex showed that SV had an identity of 92% with that of Tembusu virus and 74–89% with other viruses in the Ntaya antigen complex. Furthermore, amino acid homology of the NS5 genes of SV and Tembusu was 98.8%. The result indicated that SV was a virus molecularly very similar to Tembusu virus. However, in serological comparisons, antisera to SV showed no neutralizing activity against Tembusu virus and antisera against Tembusu virus showed weaker neutralizing activity than that of homologous virus. The results indicated that SV was a new virus which showed serological cross-reaction with Tembusu virus and also demonstrated it was a new member of Ntaya antigen complex, or clade XI of the mosquito-borne cluster in the genus Flavivirus.\(^5\) Further molec-

DISCUSSION

The Sitiawan virus isolated in this study was considered to belong to the genus Flavivirus, based on the finding that it is an RNA-type spherical enveloped virus with a diameter of approximately 41 nm, it replicates in endoplasmic reticulum and vacuoles, and its HA activity is pH dependent.\(^{13}\) The incidence of the positive reactors increased to about 70% in 5–6-week-old chicks.

However, the incidence of the positive reactors increased to about 70% in 5–6-week-old chicks.

Further molecular biological evidence indicated that SV was a very similar to Tembusu virus.
The second characteristic of clinical change was marked growth retardation. The two viral samples which have been passed in only ECE and BK3 cell cultures after ECE passages caused indistinguishable effects on the chicks. It was shown that the retardation was not a temporal effect of the viral infection since infected chicks in a group showed apparent growth retardation even at 4 weeks post-inoculation. It is, therefore, suggested that the prevalence of SV among chicks will cause economic losses to chicken farms due to low productivity.

Finally, persistent or transient increase in blood glucose levels seemed to be one of the characteristic changes, although there are few reports that describe changes of blood glucose levels in chicks after viral infection, except in cases of SMS. Due to the fact that physicochemical examinations of insulin and substances relating to the metabolism of glucose in vivo were not performed in the present study, the pathogenesis of the changes remained inconclusive. This study was initiated to disclose an etiological agent of SMS. However, none of the chicks infected with the isolated agent, SV, manifested hypoglycemia. Since occurrences of SMS were reported only in broilers, the role of SV infection in occurrences of SMS will be studied using broilers.

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REFERENCES


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† Number of positive reactors/Number of samples tested.
‡ Positive ratio.

ular biological and serological studies are required to disclose the conclusive relationship between the two viruses.

In Malaysia and often Southeast Asian countries, many flaviviruses which have unknown pathogenicity for animals have been isolated from mosquitoes, ticks, and bats, besides the wide distribution of known pathogenic flaviviruses such as JEV and Dengue viruses. Tembusu virus is one of such viruses that was isolated from mosquitoes in Kuala Lumpur in 1955. Since then, Tembusu virus has been isolated from mosquitoes of Culex species in Peninsular Malaysia, East Malaysia (Sarawak), and Thailand. High levels of serum-neutralizing antibody have been demonstrated in over 50% of man tested, although disease association with the virus has yet to be reported. Neutralizing antibody against the virus was also found in high ratios in chicks, but disease association was not known. On the other hand, it was revealed that a new isolate, SV, which has shown serological and molecular biologic relationship with Tembusu virus caused encephalitis in chicks. This fact suggests the etiological importance of Tembusu and related viruses in chicks. The high passive antibody-positive ratio and high seroconversion rate which occurred after the disappearance of passive immunity indicated the high distribution of SV and frequent occurrences of the infection among chicks. Since the virus belongs to the mosquito-borne virus cluster of flaviviruses, participation of mosquitoes is suspected in the spread of this virus. Investigation of the route of infection is required for development of preventive measures.

The virus infection was characterized by viremia which appeared in the early stages of infection, such as 1 day post-inoculation and persisted for relatively long periods, as long as 6 days. The virus was found in high levels in all tissues tested during the viremic periods. The viremia disappeared at the same time with the appearance of SN antibody. The virus titer in the brain became higher than that tested during the viremic stage, indicating virus replication in the tissue.

Encephalitis and neurological signs were the most prominent and characteristic changes in chicks infected with SV. Although the occurrence rate of clinical signs of encephalitis was not so high, most of the infected chicks showed histopathological lesions of encephalitis, such as perivascular cuffing and glial nodule formation. Further, virus distribution was high in the brain of chicks with nervous signs. These facts indicate that the virus has a strong affinity to the central nervous system and causes clinical and subclinical encephalitis in almost all infected chicks.

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Table 5

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21. Institute for Medical Research, Federation of Malaya, Annual Report. 1957. United States Army Medical Research Unit (Malaya), 100–103.