Hemagglutination and Hemagglutination Inhibition with Chuzan Virus

Yoshiyuki GOTO, Yasuo MIURA1, and Yuji KONO1

The Kyushu Branch Laboratory, National Institute of Animal Health, 2702 Chuzancho, Kagoshima 901-01 and 1National Institute of Animal Health, 3-1-1 Kannondai, Tsukuba, Ibaraki 305, Japan

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ABSTRACT. Chuzan virus agglutinated erythrocytes of several species of animals including bovine. The hemagglutinating (HA) activity against bovine erythrocytes was dependent on NaCl molarity and was expressed best at 0.6 M, but it was independent of pH and temperature. Three strains of Chuzan virus isolated from 2 cows and a pool of culicoides midges had indistinguishable HA antigenicity. All cattle infected with the virus developed high titers of hemagglutination inhibiting (HI) antibody which changed in parallel with neutralizing (NT) antibody titers. Correlation between HI and NT antibodies was very high and the antibodies persisted for one year or more. Therefore it was concluded that the HI test is applicable for survey of Chuzan virus infection among cattle in place of the NT test.—KEY WORDS: Chuzan virus, hemagglutination, hemagglutination inhibition, orbivirus.

Pregnant cows infected with Chuzan virus occasionally deliver abnormal calves with the hydranencephaly-cerebellar hypoplasia syndrome [3] and the disease is named Chuzan disease [8]. The disease was observed mainly in the Kyushu district, Japan, in 1985-1986. Although, the neutralization (NT) test has been used for survey of Chuzan virus infection among cattle and for diagnosis of Chuzan disease, development of a more simple diagnostic method, such as the hemagglutination inhibition (HI) test, was required.

It has been shown that the viruses belonging to the genus Orbivirus have hemagglutinating (HA) activity which is dependent on both the NaCl molarity and the pH of the diluent [9, 12-14]. In this study, hemagglutination (HA) and HI with Chuzan virus, which has been classified as a member of the Palyam subgroup of the genus Orbivirus [7], were investigated.

MATERIALS AND METHODS

Viruses: Three strains (K-47, C-8, and 31) of Chuzan virus isolated from the blood of healthy calves and Culicoides oxystoma [8] were used. These strains were passaged in BHK21 (baby kamter kidney cell line) cells and the K-47 strain was mainly used for this study.

Cell cultures: BHK21 cells were grown at 37°C in Eagle’s minimum essential medium (MEM) containing 10% calf serum, 10% tryptose phosphate broth (TPB) and antibiotics. The maintenance medium was MEM containing 10% TPB, 0.1% bovine serum albumin (fraction V), and antibiotics.

Preparation of HA antigen: Confluent cultures of BHK21 cells prepared in rolling culture bottles (110 × 285 mm) were inoculated with the virus at a multiplicity of infection of 0.001 and incubated with 100 ml of maintenance medium at 37°C for 7 days or longer until the cytopathic effect became complete. The culture fluid was clarified by centrifugation. When the HA titer was low, the culture fluid was concentrated by ultrafiltration. The HA antigen was stored at -80°C.

Erythrocytes: The blood of various species of animals was obtained in Alsever’s solution and stored at 4°C. The erythrocytes were used after 3 washings with physiological saline.

Bovine serum samples: Serum samples were obtained from naturally infected cattle during 1985-1986. The serum samples were stored at -20°C.

Serum treatment: Sera for the HI test were treated with kaolin to remove non-specific inhibitors and with bovine erythrocytes to absorb natural antibodies against the blood cells. One volume of test serum diluted 1:5 with VBS (0.6 M NaCl in 0.2 M Veronal buffered solution at pH 7.0) was mixed with an equal volume of 25% kaolin in VBS. The mixture was shaken at room temperature for 20 minutes and centrifuged to remove kaolin, and the supernatant was used as a 1:10 dilution of the test serum. Then, 1 ml of the serum was mixed with 100 µl of packed erythrocytes and the mixture was kept at room temperature for 30 minutes. After removal of the erythrocytes by centrifugation the serum was inacti-
vated by heating at 56°C for 30 minutes.

**HA and HI tests:** These tests were carried out by the microtiter method [12]. Bovine erythrocytes were used at a concentration of 0.3%. The diluent used was VBS containing 0.2% of bovine serum albumin.

Serial 2-fold dilutions of HA antigen were prepared in 50-μl amounts and mixed with an equal volume of erythrocyte suspension. The mixtures were incubated at 4°C overnight before the results were read. The HA titer was expressed as the reciprocal of the highest antigen dilution showing complete HA.

In the HI test, 25-μl amounts of culture fluid containing 4 units of HA antigen. After incubation at 37°C for 1 hour, 50 μl of erythrocyte suspension was added, and the results were read after incubation at 4°C for 18 hours. The HI titer was expressed as the reciprocal of the highest serum dilution showing complete HI.

**NT test:** The test was performed in microplates as described previously [7].

**RESULTS**

**Production of HA antigen in BHK21 cells:** The production of HA antigen and infective virus in the fluid phase of bottle cultures of BHK21 cells is shown in Fig. 1. Infectivity was first detected 1 day after inoculation and the titer reached a peak at 5 days. The highest HA titer was around 16. Complete cytopathic effect was observed when the HA titer reached the maximum.

**Effect of NaCl molarity, pH and temperature on HA:** The HA titer increased along with the rise in the molarity of NaCl and reached a peak at 0.6 M NaCl (Fig. 2).

No clear difference in HA activity was found in diluents having different pH's, 6.0 to 8.0, although there was a tendency that a higher HA titer was obtained at pH 6.5.

Identical HA titers were obtained when experiments were performed at 4°C, room temperature and 37°C.

**Type of erythrocytes for HA:** Bovine erythrocytes gave the highest HA titer, followed by erythrocytes from sheep, horses, rabbits and mice. Erythrocytes from guinea pigs gave a very low titer and those from hens, geese and humans were not agglutinated (Table 1).

**Comparison of antigenicility among the 3 strains of**

![Infectivity](image)

**Fig. 1.** Viral (K-47 strain) replication and hemagglutinin production in BHK21 cell cultures. ——+— indicate degrees of cytopathic effect.

![HA titer](image)

**Fig. 2.** The effect of NaCl concentration on hemagglutination by the three strains of Chuan virus.

<table>
<thead>
<tr>
<th>Erythrocytes</th>
<th>HA titer</th>
</tr>
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<tbody>
<tr>
<td>Bovine</td>
<td>64</td>
</tr>
<tr>
<td>Sheep</td>
<td>32</td>
</tr>
<tr>
<td>Horse</td>
<td>16</td>
</tr>
<tr>
<td>Rabbit</td>
<td>32</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>2</td>
</tr>
<tr>
<td>Mouse</td>
<td>16</td>
</tr>
<tr>
<td>Hen</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Goose</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Human (type O)</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

The K-47 strain was used.

**Table 1.** Hemagglutinating titers with erythrocytes from various species
**Chuzan virus**: The antigenicity was compared by the NT and HI tests. Rabbit antisera obtained after 2 repeated intramuscular inoculation with each strain of purified Chuzan virus were used. As shown in Table 2, anti K-47 serum reacted equally with the homologous and heterologous strains and the C-8 and 31 strains reacted equally with serum against the heterologous and homologous strains.

Antibody responses in cattle infected with Chuzan virus: Fifteen cows negative for NT antibody to Chuzan virus were inoculated intravenously with the K-47 strain as described in a previous paper [8] and their sera were tested for HI and NT antibodies to Chuzan virus. All the inoculated animals developed those antibodies 2 weeks after inoculation. One representative case is shown in Fig. 3. The time curves of the 2 antibodies coincided well.

Persistence of these antibodies was tested by using paired sera collected one year apart from cattle naturally infected with Chuzan virus. As shown in Fig. 4, about half of the animals kept same HI and NT titers for one year and the remaining showed a decrease in both titers to one half to one quarter.

Correlation between HI and NT antibody titers: A correlation diagram was made for NT and HI antibody titers of individual serum samples collected from adult cattle in the epizootic areas of Chuzan virus infection. As shown in Fig. 5. The HI titers were closely correlated with the NT titers ($r=0.876$).

**DISCUSSION**

HA activity of Chuzan virus, a member of the palyam subgroup of genus Orbivirus [7], was demonstrated by using bovine erythrocytes. The HA activity was dependent on NaCl molarity of the diluent, as it is for other orbiviruses (African horse sickness [9, 12], bluetongue [12], epizootic hemor-
rhagic disease [13] and Ibaraki viruses [14]). Dependence of HA activity on salt concentration has also been reported for measles virus [4, 10, 11], rabies virus [5] and Bunyaviruses [1, 2]. The mechanism by which such high salt concentrations increase HA titer is unknown, but it is possible that physical variation in the configuration of the erythrocyte surface in high salt concentrations is involved.

Three strains of Chuan virus tested, which were isolated from different hosts, two from cattle and one from culicoides midges [7], possessed identical antigenicity in NT and HI tests. All cows infected with Chuan virus produced HI antibody titers as high as those of NT antibody, the rise and fall of the HI antibody coincided with those of NT antibody, and the correlation between the two antibody titers was very high. Furthermore, the HA antigen was prepared readily in cell cultures and was very stable under frozen conditions (data not shown). These data indicate that the HI test is applicable for epidemiological surveys for Chuan virus infection among cattle and for diagnosis of Chuan disease, in place of the NT test.

REFERENCES