Application of Enzyme-Linked Immunosorbent Assay for the Seroepizootiological Survey of Antibodies against Porcine Cytomegalovirus

Tomoko TAJIMA, Takeshi HIRONAO, Taketsugu KAJIKAWA, and Hitoshi KAWAMURA

Department of Veterinary Microbiology, College of Agriculture, University of Osaka Prefecture, Sakai, Osaka 593, Japan

(Received 5 October 1992/Accepted 1 March 1993)

ABSTRACT. Enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against porcine cytomegalovirus (PCMV) was developed using the OF-1 strain of PCMV as an antigen. Results of the ELISA were compared to those of indirect fluorescent antibody (IFA) and serum neutralization (SN) tests. ELISA and IFA test were found to be extremely sensitive more than SN test. All of 11 tested sera were highly reactive in both ELISA and IFA test, but 6 of them were antibody-negative in the SN test. The retrospective survey of 436 fattening pig sera collected in Japan in 1981 showed that 433 (99.4%) of them were highly antibody-positive in ELISA.—KEY WORDS: cytomegalovirus (swine), enzyme-linked immunosorbent assay, seroepizootiological survey.


Porcine cytomegalovirus (PCMV), a member of the herpesviruses, causes inclusion body rhinitis of swine [7]. Similarly to other herpesviruses, PCMV remains in the host in latent state after primary infection, and is to be excreted in spite of the presence of circulating antibody. Infection of the herds under good sanitary management does not cause clinical symptoms, whereas infection of the susceptible herds can cause severe and even fatal disease [7]. The histopathological examination of breeding herds [10, 13] and the isolations of the virus [11, 12] indicate the PCMV infection is widespread in Japan, but serological survey has not been reported. Therefore, in this study, we tried to develop enzyme-linked immunosorbent assay (ELISA) for serological survey of PCMV infection out of 436 fattening pig sera collected from 9 districts of Japan.

MATERIALS AND METHODS

Cell and virus: A cell line (19-PFT-F) established from pig fallopian tube [5] and grown in Eagle’s minimum essential medium with 0.3% dehydrated tryptose phosphate broth, 10% calf serum and antibiotics were used. The OF-1 strain of PCMV [9] was propagated in the same manner as described previously [9].

Sera: Sera of fattening pigs were collected in the slaughter house in 44 prefectures in 9 districts of Japan, except Oita, Miyazaki and Kagoshima in Kyushu district, in 1981. Reference positive serum against PCMV was obtained from gnotobiotic pig infected with PCMV [11]. Sera from 3 gnotobiotic pigs were also used as the negative control in the study.

Preparation of ELISA antigen: The ELISA antigen was prepared from the 19-PFT-F cells after 2 to 10 days post inoculation (DPI) with PCMV. Confluent 19-PFT-F cells were inoculated with 10⁴.5 50 percent tissue culture infective dose per ml (TCID₅₀/ml) of PCMV, OF-1 strain. The infected cells were solubilized with reticulocyte standard buffer (0.01 M NaCl, 0.0015 M MgCl₂, 0.01 M Tris-HCl, pH 7.4) containing 0.2% Nonidet P-40 at room temperature for 20 min, and centrifuged at 2,000 rpm for 5 min. The supernatant was filtrated through filter of 300 nm pore size and used as the antigen. Uninfected 19-PFT-F cells were treated in the same manner as described above and used as negative control antigen. The antigenic activity of the prepared antigen was examined in the ELISA using 1:100 dilution of the anti-PCMV serum. Protein concentration of the PCMV antigen was assayed with Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.). The antigens were stored at −80°C until use.

ELISA technique: The PCMV antigen or negative control antigen diluted with 0.05 M carbonate buffer, pH 9.6, was absorbed to individual wells of microplates and left overnight at 4°C. Antigen excess was removed after absorption, and the wells were postcoated with 100 μl of a phosphate buffered saline (PBS) containing 25% of Block Ace (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) for 60 min at 37°C to block the nonspecific protein absorption sites. Test sera diluted from 1:100 to 1:12,800 were then added to PCMV antigen or negative control antigen coated well (50 μl/well) and incubated for 60 min at 37°C. After washing 3 times with 0.05% Tween 20 in PBS, 50 μl of a predetermined concentration (1:500) of peroxidase-conjugated anti-swine IgG rabbit serum (Cappel, West Chester, PA, U.S.A.) was added to each well and allowed to react 30 min at 37°C. Reagent excess was removed by washing as described above, and 0.2 mM 2,2-azino-bis(3-ethylbenzthiazoline sulfonylic acid) (ABTS) containing 0.004% H₂O₂ in 0.05 M citrate buffer, pH 4.0, was added as substrate. The plate was then incubated at room temperature for 20 min and the absorbance at 405 nm was measured. The results were calculated as the absorbance obtained by the subtracted absorbance of the PCMV antigen-coated wells by those of the normal antigen-coated wells. The subtracted absorbance was higher than 0.1, shown to be positive.

Indirect fluorescent antibody (IFA) test: The 19-PFT-F monolayer cells were infected with 10⁴.5 TCID₅₀/ml of the
PCMV, OF-1 strain, and were cultured for 4 days. The infected cells were then dispersed with trypsin and mounted on the well of the teflon-coated multistest slides (Flow Labs. Inc., McLean, VA, U.S.A.), and fixed in acetone. The slides were dried and stored at -20°C until use. Test sera were titrated with twofold dilution ranging from 1:10 to 1:5120. Diluted sera were placed on the wells of antigen slides and incubated for 1 hr at 37°C. The slides were then washed with PBS and dried. Each well was covered with fluorescein isothiocyanate-conjugated anti-swine IgG rabbit serum (Cappel, West Chester, PA, U.S.A.) diluted 1:40 and the slides were again incubated for 1 hr at 37°C. After washing with PBS, the samples were examined with fluorescent microscope.

Serum neutralization (SN) test: Test sera diluted with two fold serial dilution was mixed with an equal volume of PCMV, OF-1 strain, containing 10³ TCID₅₀/mL, and incubated at 37°C for 1 hr. The mixture was then inoculated on the 19-PFT-F monolayer cells seeded onto microplate wells. After 10 days cultivation, results were read and the SN titer was expressed as the reciprocal of the highest dilution of serum which neutralized virus in more than 50% of the well [9].

RESULTS

ELISA antigen: The antigenic activity of the ELISA are shown in Table 1. Since the antigen made from the cells of 7 DPI to 10 DPI showed positive reaction and the antigen of 10 DPI had the highest absorbance, the cells of 10 DPI were used for preparation of the antigen. The optimal antigen dilution to be used was determined by checkerboard titration using a positive and negative serum diluted from 1:100 to 1:12,800. The protein concentration of the antigen at 1:100 dilution was 30 μg/ml and 4 unit of the antigen, 1:100 dilution was enough to detect the antibody (data are not shown).

Comparison of ELISA, IFA test, and SN test: Sensitivity of ELISA, IFA and SN tests for the detection of PCMV antibody were compared (Table 2). As the results, the negative control gnotobiotic pig serum showed negative in all tests. All of 11 sera tested were positive and were highly reactive in ELISA and IFA test, but 6 of them were found to be negative in SN test. All positive sera except one were 2.5-5 fold or more higher in titer by ELISA than in those by IFA. The coefficient of correlation of titers between ELISA and IFA test were 0.616 and were statistically significant (p<0.05), however, those between ELISA and SN test were 0.195 and were not significant.

Survey of the antibodies against PCMV in pig sera: Four hundred and thirty six sera collected from the fattening pigs in 44 prefectures were surveyed by ELISA. The results were arranged and summarized in each district (Table 3). Of them, 433 (99.4%) were positive and the ELISA titers of these positive sera varied from 400 to higher than 12,800. Of 319 positive sera had higher antibody titer more than 1:12,800 in the ELISA. No difference in the incidence among the districts or the

<table>
<thead>
<tr>
<th>Days post inoculation with PCMV</th>
<th>Dilution of the antigen(a)</th>
<th>Absorbance at 405 nm(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt;10</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>2</td>
<td>&lt;10</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>3</td>
<td>&lt;10</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>4</td>
<td>&lt;10</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>5</td>
<td>&lt;10</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>6</td>
<td>&lt;10</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>7</td>
<td>≥1280</td>
<td>0.108</td>
</tr>
<tr>
<td>8</td>
<td>≥1280</td>
<td>0.170</td>
</tr>
<tr>
<td>9</td>
<td>≥1280</td>
<td>0.168</td>
</tr>
<tr>
<td>10</td>
<td>≥1280</td>
<td>0.219</td>
</tr>
</tbody>
</table>

Negative control <10 0

a) Titers expressed as the reciprocal of the highest dilution showed the positive reaction.
b) Absorbance at the highest antigen dilution showed the positive reaction. Absorbance of each antigen was subtracted by that of the negative control.

Table 1. Antigenic activity of the cells inoculated with porcine cytomegalovirus

<table>
<thead>
<tr>
<th>Serum</th>
<th>Antibody titer(a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gnotobiotic pigs</td>
<td></td>
</tr>
<tr>
<td>PCMV-non infected</td>
<td>&lt;10</td>
</tr>
<tr>
<td>PCMV-infected</td>
<td>5120</td>
</tr>
<tr>
<td>Conventional pigs(b)</td>
<td></td>
</tr>
<tr>
<td>No. 1</td>
<td>5120</td>
</tr>
<tr>
<td>No. 2</td>
<td>2560</td>
</tr>
<tr>
<td>No. 3</td>
<td>5120</td>
</tr>
<tr>
<td>No. 4</td>
<td>5120</td>
</tr>
<tr>
<td>No. 5</td>
<td>2560</td>
</tr>
<tr>
<td>No. 6</td>
<td>640</td>
</tr>
<tr>
<td>No. 7</td>
<td>2560</td>
</tr>
<tr>
<td>No. 8</td>
<td>640</td>
</tr>
<tr>
<td>No. 9</td>
<td>640</td>
</tr>
<tr>
<td>No. 10</td>
<td>2560</td>
</tr>
</tbody>
</table>

a) Titers expressed as reciprocal of the highest dilution of sera. b) Sera collected in Fukui prefecture.

preference (data are not shown) was observed. Three (0.6%) negative sera in ELISA were also negative in the IFA test using 1:10 dilution (data are not shown).

DISCUSSION

The purpose in this study was to develop the ELISA for detection of antibodies against PCMV and to survey the antibody to PCMV in fattening pigs in Japan.

The ELISA developed in this study for the detection of PCMV antibody has to be specific. The antibody titers of the test sera obtained in ELISA were higher than that in IFA and SN tests. But the coefficient of correlation of titers between ELISA and IFA test was statistically
Table 3. Distribution of porcine cytomegalovirus-antibody in fattenings pigs in Japan

<table>
<thead>
<tr>
<th>District</th>
<th>Antibody titer</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≥12800</td>
<td>6400</td>
</tr>
<tr>
<td>Hokkaido</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Tohoku</td>
<td>47</td>
<td>7</td>
</tr>
<tr>
<td>Kantō</td>
<td>72</td>
<td>5</td>
</tr>
<tr>
<td>Chubu</td>
<td>69</td>
<td>13</td>
</tr>
<tr>
<td>Kinki</td>
<td>35</td>
<td>15</td>
</tr>
<tr>
<td>Chugoku</td>
<td>36</td>
<td>6</td>
</tr>
<tr>
<td>Shikoku</td>
<td>26</td>
<td>4</td>
</tr>
<tr>
<td>Kyushu</td>
<td>24</td>
<td>8</td>
</tr>
<tr>
<td>Okinawa</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

| Total (%) | 319 | 63 | 41 | 8 | 1 | 1 | 3 | 436 |

|           | (74) | (14) | (9) | (2) | (0.2) | (0.2) | (0.6) |

a) Number of pig.

significant, and the antibody-negative sera in IFA test were also negative in ELISA. Antibody titers in ELISA were relatively high and varied from 1:400±1:12,800. Assaf et al. [3] also developed the ELISA for the detection of PCMV antibodies and reported that 9.54% of the sera tested had antibody titers from 1/20 to 1/640. The ELISA titers they reported were very low when compared with those of our results. However, there are some differences in our experiment and theirs. The infected cells as the ELISA antigen was sonicated in their experiment, but was solubilized with detergent in ours. And also they used 5-amino salicylic acid as a substrate in ELISA and read the results visually, but we used ABTS as a substrate and measured the absorbance of each well. These differences in both technique and procedures of methods may produce the differences in sensitivity of the ELISA.

In the SN test, six of ten sera were negative, but they were highly reactive in the ELISA and IFA test. It was reported that the existence of certain antigenic differences in virus neutralizing activity of human cytomegalovirus strains [1, 2] and in indirect membrane-immunofluorescence [8]. The titer in sera against the heterologous strain was much lower than the homologous strain [1, 2, 8]. Antigenic differences between the strains and the serological methods which might be of importance for the serodiagnosis of PCMV infection. In the present study, we used only the OF-1 strain of PCMV, and the method of SN test used in our study is different from others [1, 2]. Glycoprotein of pseudorabies virus play a significant role in eliciting neutralizing antibody in infected animals [4]. The protein analysis of PCMV related with antigenic variation is also needed.

Present study showed that the PCMV infection was already widespread as early as 1981 in Japan and the latent infection might be established in these pigs. It was pointed out that the latent virus is usually the infectious source in cytomegalovirus-related illness during immunosuppression or immunodeficiency. Though the outbreaks of inclusion body rhinitis are rare, mixing of new stock for breeding is always a potential source of PCMV mainly through nasal shedding and may relative latent PCMV [6]. It remains also important to investigate the participation of PCMV to the infection of the chronic respiratory diseases and/or other chronic diseases.

ACKNOWLEDGMENT. We thank Dr. H. Kodama, Department of Veterinary Immunology, College of Agriculture, University of Osaka Prefecture, for his critical reading of the manuscript.

REFERENCES

