Isolation of Antigenic Variants of Leptospiras from Puppies and Pigs Experimentally Infected with *Leptospira interrogans* Serovars *canicola* and *pomona*

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**Abstract.** For the purpose of isolating antigenic variants of leptospiras from domestic animals, *Leptospira interrogans* serovars *canicola* and *pomona* were inoculated into puppies and specific-pathogen-free (SPF) pigs respectively, and the blood and kidneys of those animals were inoculated onto the solid medium each containing homologous immune serum. Colonies developed on the medium were divided into large, medium and small colonies. Some of these colonies picked at random were antigenically compared with the parent by a screening test using the precipitin-absorption test in gel. In the case of *canicola*, the antigenic variants were isolated from the blood of 7 of the 10 infected puppies. The antigenic variants were found in a large number of the large colonies from the blood and kidneys, and in a small number of the medium and small colonies from the blood, and none of the medium and small colonies from the kidneys. The antigenic variants were also isolated from the inocula, but they were isolated more frequently from the blood of the puppies than from the inocula. In the case of *pomona*, the antigenic variants were isolated only from the small colonies from the blood of 2 of the 5 infected pigs. Large colonies were never developed from the specimens of the pigs. The antigenic variants were isolated more frequently from the blood of the pigs than from the inocula. The variants were confirmed to be antigenically different from their parent. The variants originated from the same parent were antigenically similar to one another. These results indicate that *canicola* Moulton and *pomona* MLS contained a small percentage of the antigenic variants and that the percentage of the variants was raised significantly, 3 to 7 days after the infection, in the blood of the puppies and pigs.

Antigenic variants of leptospiras were isolated from the kidneys of mice inoculated with *Leptospira interrogans* serovar *copenhageni* strain Shibaura [4] and from the blood of the guinea pigs inoculated with the cloned culture of same strain [6]. It was found in these studies that leptospiras formed small colonies on the solid serum medium containing the appropriate amount of the homologous immune serum while the antigenic variants from the same strain produced large colonies on the same medium [4, 6, 8]. The concentration of the immune serum in the medium was within the range that the number of the colonies of leptospiras was not decreased. The solid medium containing the homologous immune serum was, therefore, thought to be helpful to detect the antigenic variants of leptospiros [6].

If antigenic variants appear in domestic animals, it will be important from the viewpoint of epidemiology, prevention and serological diagnosis of leptospirosis. Attempts were made in the present study to isolate antigenic variants of leptospiras...
from puppies and pigs experimentally infected with serovars canicola and pomona respectively. The results obtained are described below.

**Materials and Methods**

**Strains:** The strains used were canicola Moulton and pomona MLS, both were hamster lethal and provided by R. C. Johnson, University of Minnesota, Minneapolis, Minnesota. Prior to the experiments, canicola Moulton passed once through a puppy; the cardiac blood of the puppy obtained 5 days after the exposure was cultivated in the normal serum medium and used for experiments. Pomona MLS passed through hamster once and subcultured twice in the normal serum medium was used for experiments.

**Animals:** A total of 24 mongrel puppies of both sex, ranging in age from 1 to 3 weeks and weighing from 260 to 670 g was used. Half of the puppies were used for the inoculation experiment, and the remaining half for control. Before being used each puppy was proved to be serologically negative (at 1:10 serum dilution) against canicola Moulton by examining the serum from the cardiac blood by means of the microscopic agglutination test.

Five SPF pigs (Koizumi farm, Shizunai, Hokkaido) at the age of 3 weeks weighing from 5 to 10 kg were used for inoculation of pomona MLS. They were serologically negative (at 1:10 serum dilution) before use in the microscopic agglutination test using the following antigens, pomona Pomona, icterohaemorrhagiae RGA, canicola Hond Utrecht IV, autumnalis Akiyami A, hebdomadis Hebdomadis, australis Ballico and pyrogenes Salinem.

**Serum medium:** A 0.2% tryptose phosphate broth (Difco) containing 9% normal rabbit serum was the normal serum medium and the serum medium solidified with 1% agar (Special agar Noble, Difco) [1] was the solid normal serum medium.

**Immune serum medium:** Each rabbit was hyperimmunized with either canicola Moulton or pomona MLS, following the method described before [7]. The titer of the each immune serum against the homologous antigen, expressed in 50% agglutination end-point, was 1:30000. The solid normal serum medium containing the homologous immune serum, either 0.2% of the anti-canicola Moulton serum or 0.1% of the anti-pomona MLS serum, was used as the solid immune serum medium.

**Inoculation of leptospirosis:** Puppies were inoculated with the 7–10 day-old cultures of canicola Moulton, either $7 \times 10^5$–$9 \times 10^8$ organisms intracardially or $4 \times 10^4$–$2 \times 10^6$ organisms subcutaneously. The number of organisms was determined using a counting chamber [5]. Uninoculated puppies were used as control.

Pigs were inoculated with the 7–10 day-old cultures of pomona MLS $10^9$ organisms in the anterior vena cava.

Isolation of antigenic variants: The blood was taken at the febrile stage either via the heart of puppies or via the jugular of pigs, and was immediately diluted in a 1:5 ratio with M/100 phosphate buffer solution, pH 7.2 (PBS); 0.2 ml of this preparation or serial tenfold dilution of the preparation was inoculated onto the solid immune serum medium.

The kidney cortex of the infected puppies was cut into small pieces approximately 1 mm², and immersed in 2 ml of PBS; 0.2 ml of fluid part or serial tenfold dilution of the fluid part was inoculated onto the solid immune serum medium.

Several parts of the kidney cortex of the pigs, 1 g amount, were ground in 3 ml of PBS, and 0.2 ml of the preparation and its serial tenfold dilution was inoculated onto the solid immune serum medium.

The inocula were examined immediately after inoculating into animals whether or not they contained the antigenic variants. For this purpose, 0.2 ml of tenfold dilutions of the inoculum was inoculated onto the solid immune serum medium.

The plates inoculated with samples from the puppies and pigs were incubated for 2 to 5 weeks, and those inoculated with samples from the inoculum were for 2 to 3 weeks at 30°C.

Of the colonies developed on the solid immune serum medium, the large colonies were presumed to be antigenic variants [4], some of the typical large, medium and small colonies were picked at random into the serum medium respectively. Sodium dodecyl sulfate (SDS)-extracted antigen was prepared from each clone, as described before [7]. Whether the antigenicity of each clone was different from that of the parent was examined as follows, as shown in Fig. 1, by the precipitin-absorption test [6]. 1) Well PS-D45-L1 was filled with the antigen of a clone to be examined, which was D45-L1 isolated from the blood of a puppy. After drying the antigen at room temperature the well was filled again with the same antigen and dried. The antiserum (canicola Moulton) serum (usually 1:2) was then added to the well. 2) The antigen of the parent and the clone were filled in wells P (upper left) and D45-L1, respectively. 3) In another set of wells, PS 1:10 was filled with the anti-parent serum (1:10) and P (upper right) was filled with the homologous
antigen. 4) 3–5 days later, the tests were read. If the precipitin line which formed between wells PS-D45-L1 and P is stronger than the line which appeared between wells PS 1:10 and P, the clone D45-L1 was considered to be an antigenic variant. No reaction was seen between PS-D45-L1 and D45-L1.

The isolated clones were maintained by passage through the normal serum medium at intervals of 4 to 6 weeks.

Microscopic agglutination, immunodiffusion and precipitin-absorption test in gel: These tests were done as previously described [3, 7].

Agglutinin absorption test: The test was done according to Kmety et al. [2].

Results

1. A preliminary experiment on the concentration of anti-canicola Moulton serum to be added to the solid serum medium

Two hundred and forty leptospirosis, counted using the chamber, were inoculated on the solid medium containing 0.5, 0.3, 0.2 and 0.1% of the homologous immune serum. The same amount of leptospirosis were inoculated on the solid medium containing no immune serum.

The number of colonies developed on 20th day were 97, 103, 92, 87 and 62 on the solid medium containing 0, 0.1, 0.2, 0.3 and 0.5% of the immune serum. The diameter of the colonies was decreased in inverse proportion to the percentage of the immune serum. The diameter of the colonies was 5–20 mm on 20th day on the normal serum medium and only 0.1 mm on 29th day on the medium containing 2% of the immune serum. The number of the colonies developed were not different in the solid medium containing 0.2, 0.1 and 0% of the immune serum (P<0.01). Therefore, 0.2% of the immune serum were
Table 1. Isolation of antigenic variants from blood of puppies inoculated with canicola Moulton

<table>
<thead>
<tr>
<th>Inoc. route&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Inoc. size</th>
<th>Pup. No.</th>
<th>Days after inoc.</th>
<th>No. of colonies</th>
<th>No. of variants&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Large</td>
<td>Medium</td>
</tr>
<tr>
<td>IC</td>
<td>7X10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>24</td>
<td>4</td>
<td>173</td>
<td>537</td>
</tr>
<tr>
<td>&quot;</td>
<td>4X10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>39</td>
<td>4</td>
<td>28</td>
<td>31</td>
</tr>
<tr>
<td>&quot;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>60</td>
<td>4</td>
<td>3</td>
<td>64</td>
<td>94</td>
</tr>
<tr>
<td>&quot;</td>
<td>43</td>
<td>5</td>
<td>8</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>&quot;</td>
<td>46</td>
<td>5</td>
<td>13</td>
<td>39</td>
<td>10</td>
</tr>
<tr>
<td>&quot;</td>
<td>45</td>
<td>6</td>
<td>2</td>
<td>38</td>
<td>0</td>
</tr>
<tr>
<td>&quot;</td>
<td>9X10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>26</td>
<td>4</td>
<td>51</td>
<td>8</td>
</tr>
<tr>
<td>SC</td>
<td>4X10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>37</td>
<td>7</td>
<td>9</td>
<td>204</td>
</tr>
<tr>
<td>&quot;</td>
<td>8X10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>61</td>
<td>6</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>&quot;</td>
<td>2X10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>52</td>
<td>3</td>
<td>1</td>
<td>62</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
<td>287</td>
<td>997</td>
</tr>
</tbody>
</table>

<sup>a</sup> IC, intracardially; SC, subcutaneously.

<sup>b</sup> No. of antigenic variants/No. of colonies examined.

<sup>c</sup> Not tested.

<sup>d</sup> Not applicable.

added to the solid medium thereafter.

2. Isolation of the antigenic variants from the blood of puppies inoculated with canicola Moulton

Of the 12 puppies, 10 showed fever of more than 39°C for 3–7 days after the inoculation. They also showed diarrhea, vomiting and dehydration. The cardiac blood of the puppies, obtained at the febrile stage and inoculated onto the solid immune serum medium, produced large, medium and small colonies (Fig. 2 and Table 1). The sizes of colonies could be differentiated on the 16–22 days of incubation. The large colonies were more than 2 mm, the medium colonies were 0.5–2 mm and the small colonies were less than 0.5 mm in diameter. The number of colonies developed and the ratio of the three sizes colonies were different in puppies. Generally, the large colonies were small in number, while the medium and the small colonies were large in number.

The colonies selected at random were examined to determine whether they were antigenically different from the parent. Fig. 1 point out an example, as mentioned in Materials and Methods; D45-L1, a clone from puppy No. 45, is a variant. The number of the antigenic variants appeared in each puppy is shown in Table 1.

The antigenic variants were isolated from the blood of 7 of the 10 puppies. In total, the variants were 19 of the 40 large colonies while 3 of the 20 and 2 of the 40 medium and small colonies, they appeared more frequently in the large colonies than in the medium and the small colonies.

Agglutinin antibody titer against canicola Moulton at the febrile stage (6th post-infection day) was examined only in puppy No. 45; it was 1:100. The seroconversion was thus proved to be positive in the puppy.

3. Isolation of the antigenic variants from the kidney of the puppies inoculated with canicola Moulton

The kidney cortex of 7 of the 12 puppies, obtained 2–6 days after the inoculation, produced on the solid immune serum me-
ANTIGENIC VARIANTS OF LEPTOSPIRAS

Table 2. Isolation of antigenic variants from kidney of puppies inoculated with canicola Moulton

<table>
<thead>
<tr>
<th>Inoc.</th>
<th>Inoc.</th>
<th>Pup.</th>
<th>Days after Inoc.</th>
<th>No. of colonies</th>
<th>No. of variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>route</td>
<td>size</td>
<td>No.</td>
<td>Large</td>
<td>Medium</td>
<td>Small</td>
</tr>
<tr>
<td>IC</td>
<td>4×10^7</td>
<td>43</td>
<td>5</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>&quot;</td>
<td></td>
<td>43</td>
<td>5</td>
<td>3</td>
<td>301</td>
</tr>
<tr>
<td>&quot;</td>
<td></td>
<td>60</td>
<td>5</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>&quot;</td>
<td>7×10^7</td>
<td>32</td>
<td>2</td>
<td>6</td>
<td>74</td>
</tr>
<tr>
<td>&quot;</td>
<td></td>
<td>30</td>
<td>3</td>
<td>1</td>
<td>95</td>
</tr>
<tr>
<td>&quot;</td>
<td>9×10^7</td>
<td>26</td>
<td>5</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>SC</td>
<td>8×10^7</td>
<td>61</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>15</td>
<td>491</td>
<td>71</td>
</tr>
</tbody>
</table>

a IC, intracardially; SC, subcutaneously.
b No. of antigenic variants/No. of colonies examined.
c Not applicable.
d Not tested.

Table 3. Isolation of antigenic variants from the inocula of canicola Moulton

<table>
<thead>
<tr>
<th>Exp.</th>
<th>No. of colonies</th>
<th>No. of variants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Large</td>
<td>Medium</td>
</tr>
<tr>
<td>I</td>
<td>19</td>
<td>564</td>
</tr>
<tr>
<td>II</td>
<td>133</td>
<td>3591</td>
</tr>
<tr>
<td>III</td>
<td>39</td>
<td>558</td>
</tr>
<tr>
<td>IV</td>
<td>75</td>
<td>220</td>
</tr>
<tr>
<td>V</td>
<td>84</td>
<td>939</td>
</tr>
<tr>
<td>VI</td>
<td>51</td>
<td>558</td>
</tr>
<tr>
<td>Total</td>
<td>401</td>
<td>9430</td>
</tr>
</tbody>
</table>

a No. of antigenic variants/No. of colonies examined.
b Not tested.

dium, large, medium and small colonies, as in the cases of the blood. The antigenic variants were exclusively the large colonies and were isolated from the kidneys of 3 puppies (Table 2). No leptospiras were isolated from the blood and the kidneys of uninoculated puppies.

4. Isolation of the antigenic variants from the inocula of canicola Moulton

A portion of the inoculum, which was the culture in the normal serum medium, was inoculated on the solid immune serum medium immediately after the inoculation into puppies. In total of 6 experiments, 401 large, 6430 medium and 862 small colonies were developed. Ten of the 28 large colonies, none of the 16 medium colonies and 3 of the 28 small colonies were found to be antigenic variants (Table 3).

5. Comparison of frequency of the antigenic variants from the blood and kidneys of the puppies and the inocula of canicola Moulton

The percentage of the antigenic variants in all the colonies from the blood and kidneys of the puppies and the inocula of canicola Moulton was estimated from the results shown in Tables 1, 2 and 3. The percentage of the variants was calculated at 14.6% of the colonies from the blood, 1.8% of the colonies from the kidneys and 3.1% of the colonies from the inocula. The percentage of the variants in the colonies from the blood was significantly higher than that in the colonies from the kidneys (P<0.005, χ^2-test) and than that in the colonies from the inocula (P<0.005, χ^2-test).

6. Serological characterization of the variants of canicola Moulton by agglutinin-
absorption test and precipitin-absorption test in gel.

The cross agglutinin-absorption test was done using the immune sera against the parent and the variant D45-L1. It is obvious from the agglutinin-absorption test that the variant D45-L1 is antigenically different from the parent (Table 4). With the limitations of the test procedure, other variants, D45-L2, D52-M, D52-S (isolated from the blood of puppy Nos. 45 and 52) and Moul-L1 (from the inoculum of canicola Moulton) were different from the parent and similar to D45-L1. These results agreed with those of the cross precipitin-absorption test using anti-parent and anti-D45-L1 sera. Figs. 3 and 4 show the test using anti-parent serum and the serum absorbed with D45-L1.

7. Isolation of antigenic variants from the blood and kidneys of pigs inoculated with *pomona* MLS

The concentration of anti-*pomona* MLS serum to be added to the solid serum medium was determined, as in the case of anti-canicola Moulton serum, and 0.1% of the anti-*pomona* MLS serum were found to be added to the solid serum medium.

Five SPF pigs showed fever of more than 40°C 3–5 days after the inoculation, and 3 showed loss of appetite. The blood of the pigs at the febrile stage was inoculated onto the solid immune serum medium. Medium and small colonies but not large colonies were developed from 17th to 20th days of incubation from the 4 pigs (Table 5). No colonies were developed from the blood of the remaining pig. The medium colonies were 0.5–2 mm and small colonies were less than 0.5 mm in diameter. The colonies of *pomona* MLS were, however, always much hazier than those of canicola Moulton.

The antigenic variants were isolated from the blood of 2 pigs. One of the pigs produced only small colonies. The antigenic variants from the blood of these pigs were found only in the small colonies.

Only a small number of medium and small colonies grew from the kidneys of 2 pigs which were examined 17 and 24 days after the inoculation. None of them were antigenic variants.

Agglutinin antibody titers against *pomona* MLS at the febrile stage were 1:10, 1:30 and 1:300 in pig Nos. 3, 4 and 5 respectively, and less than 1:10 in Nos. 1 and 2.

8. Isolation of the antigenic variants from the inocula of *pomona* MLS

Antigenic variants in the inocula of *po-

<table>
<thead>
<tr>
<th>Immune serum against</th>
<th>Absorbed with</th>
<th>Agglutinin titer with antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>canicola</td>
<td>D45-L1</td>
</tr>
<tr>
<td>canicola</td>
<td>Nil</td>
<td>30000&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>D45-L1</td>
<td>30000</td>
</tr>
<tr>
<td></td>
<td>Nil</td>
<td>3000</td>
</tr>
<tr>
<td>D45-L1</td>
<td>canicola</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>D45-L1</td>
<td>—</td>
</tr>
</tbody>
</table>

<sup>a</sup> Variants D45-2, D52-M, D52-S and Moul-L1.
<sup>b</sup> Reciprocal of the highest dilution showing 50% or more agglutination.
<sup>c</sup> Dashes represent negative reaction at 1:100 dilution.
mouna MLS, which was the culture in the normal serum medium, were examined. In total, 1 large (3.6 mm in diameter), 892 medium and 2134 small colonies were developed. This large colony, but none of the 16 medium and the 20 small colonies were found to be the antigenic variants (Table 6).

9. Comparison of frequency of the antigenic variants from the blood of the pigs and the inocula of pomona MLS

The percentage of the antigenic variants in all the colonies from the blood of the pigs and the inocula of pomona MLS was estimated from the results shown in Tables 5 and 6. The percentage of the variants was calculated at 8% of the colonies from the blood and 0.3% of the colonies from the inocula. The percentage of the variants in the colonies from the blood was higher than that in the colonies from the inocula (P<0.005, χ²-test).

10. Serological characterization of the variants of pomona MLS by agglutinin-absorption test and precipitin-absorption test in gel

The cross agglutinin-absorption test was done using the immune sera against the
parent, the variant P2-S1 (isolated from the blood of pig No. 2) and the variant MLS-L (isolated from the inoculum of *pomona* MLS). The variants P2-S1 and MLS-L were similar each other, and were different from the parent by the agglutinin-absorption test (data were not shown). With the limitations of the test procedure, other variants, P1-S and P2-S2 (from the blood of the pig Nos. 1 and 2) were different from the parent and similar to P2-S1 and MLS-L. The results of the cross precipitin-absorption test in gel agreed with the results of the agglutinin-absorption test.

11. The antibody against the antigenic variants developed in pigs

Whether or not the infected pigs produce the antibody against the antigenic variant, 3 pigs which survived for 24 to 42 days after the inoculation were examined. To detect the antibody against the variant the sera absorbed with *pomona* MLS were examined with the antigen of P2-S1. The antibody against the variant was undetectable before inoculation but was detected in 2 pigs 14 days after the inoculation. The titer was 1:100. Table 7 shows the agglutinin titer of pig No. 2.

12. Antigenic stability of the variants

Antigenicity of the variants was examined after 3 to 6 passages, at intervals of 4 to 6 weeks, in the normal serum medium. Of the 31 variants of *canicola* Moulton 30 were found to have reverted to their original state. Variant D45-L1 (from the blood of puppy No. 45) was the only variant which retained the antigenicity after the 6th passage which was the highest passage in this experiment. Of the 4 variants of *pomona* MLS, 3 were reverted after 4 passages to their original state, while a remaining variant MLS-L (isolated from the inoculum) retained its antigenicity after the 6th passage.

Discussion

It was found in the preceded work that the antigenic variants of leptospiras were differentiable from the parent on the solid immune serum medium by their tendency to form large colonies [6]. In the present study, therefore, the solid immune serum medium was used to facilitate the isolation of antigenic variants. There might be a possibility that the leptospiral cell antigenically different from the parent secondarily appear while each parental colony is being formed on the solid immune serum medium. However, the size of such colonies can not be large; they are scored as the small colonies.

It was shown that antigenic variants of leptospiras were isolated not only from the mice inoculated with *copenhageni* [4] and the guinea pigs inoculated with a clone of the same strain [6] but also from the dogs and pigs inoculated with *canicola* and *pomona* respectively. These findings may be helpful in order to grasp a new character of leptospiras that is antigenically considerably variable in vivo.
Canicola Moulton and pomona MLS were found to contain a small percentage of the antigenic variants, and the percentage of the variants was raised significantly, 3 to 7 days after infection, in the blood of the puppies and pigs. Perhaps a small amount of antibodies appeared in the blood of the animals after infection exerted some selective effect on the antigenic variants. These findings agree with those of the previous experiment using clonized copenhageni and guinea pigs [6].

The percentage of the variants was raised in the blood but not in the kidneys. The reason of this finding is not clear. Leptospiras might reached, before the antibody appeared in the blood, to the renal tubuli, where they survived avoiding the effect of the antibody. However, the leptospiiral colonies recovered from the kidneys were small in number, and we were obliged to examine only such a small number of colonies.

It was already shown that 1.2% of leptospiiral cells were the antigenic variants in the culture of clonized copenhageni Shibaura [6]. The presence of the antigenic variants in the clonized culture made us in the present study to use unclonized strains. The percentage of the antigenic variants in the cultures of canicola Moulton and pomona MLS, which were used as the inocula, was 2.9% and 0.3% respectively. The percentage of the variants in the cultures of the two strains were somewhat similar to that in the culture of clonized copenhageni Shibaura.

It was presumed that the variants were distinguishable from the parent as they produce large colonies on the solid immune serum medium, as in the case of the variants of copenhageni which were isolated from the blood of the experimentally infected guinea pigs [6]. Although variants tended to form large colonies in the present study, the size of colonies did not always correlate with the antigenic variation.

The variants of canicola Moulton were antigenically similar one to another. This was also the case in the variants of pomona MLS. The antigenic variation in each strain seems to tend to proceed in a direction, under a certain pressure such as the presence of the homologous immune serum.

The antibody against the antigenic variant of pomona was detected after absorbing anti-parent agglutinin, in 2 pigs. Although the agglutinin titer against the variant was only 1:100 on 14th post infection day, the appearance of the anti-variant antibody in pigs is interesting.

Two types of the antigenic variants were isolated in the present study: 1) The variants which were antigenically stable. 2) The variants which reverted to their original state after several passages in the normal serum medium. These types of variants were noticed only in the latter part of the experiment, each type of the variants was not thoroughly examined. However, stable variants were small in number, while unstable variants were large in number. The stable variants may be the antigenic mutants corresponding to those appeared while leptospiras were being cultivated in the liquid immune serum medium [4, 8]. The unstable variants on the other hand, may have appeared due to adaptation.

The unstable variants were noticed for the first time in the present study. Perhaps the use of solid immune serum medium and examination of many colonies of various sizes revealed the existence of such unstable variants. Nature of the unstable variants is a subject of future study.

We feel that the antigenic variation, either mutation or adaptation, may be able
to demonstrate the ability of leptospiras to survive in a harmful environment, in the presence of antibody.

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