Immunization with Rabies Inactivated Vaccine for animal use (PV2061 Strain) elicits potent immune responses and protects dogs against lethal challenge

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ABSTRACT
Despite the availability of efficacious vaccines for animals and humans, rabies is still a major zoonosis. Prevention of rabies in dogs is the key for reducing the risk of transmission of this deadly disease to humans. The protection of immunized animals was largely dependent on the induced humoral immune response. The objective of this study was to validate the rabies inactivated vaccine, PV2061Strain on one vaccination. 14000 dogs of different ages were involved in this research. The result showed that the vaccinated dogs were induced a strong and sustained antibody response and almost had no clinical manifestations. What is more, all vaccinated animals (30 dogs selected randomly from vaccinated dogs) were protected against virulent rabies challenge carried out 1 year after vaccination. These results validated 1 year duration of immunity after a vaccination with PV2061.

Introduction
Rabies virus (RABV), which belongs to the genus Lyssavirus of the family Rhabdoviridae, causes a fatal neurologic disease in humans and animals [1]. Approximately 55,000 human deaths caused by rabies are reported annually, with most of these cases occurring in.

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developing countries\[2\].

Stray dogs, wild carnivores, and bats are the natural reservoirs of field RABV, and these rabid carriers are a public health risk to humans and domestic animals. Human rabies occurrence is largely attributed to the bite of stray dogs in developing countries, where vaccination of animals is limited, especially in rural areas\[3-5\].

Vaccination is the most effective method of preexposure treatment against RABV infection and has been used in both humans and reservoir animals. In the developed countries, human rabies has been eliminated or reduced to a minimum due to rabies control programs during the past 60 years (routine and mass vaccination of dogs) \[6\]. The use of live, attenuated vaccines is no longer recommended for parenteral immunization in animals by the World Health Organization (WHO) Expert Consultation on Rabies \[7, 8\]. With the exception of the canarypox-virus vectored vaccine against rabies, all rabies vaccines currently marketed for companion animals are inactivated adjuvanted vaccines. Inactivated vaccines are the most commonly used anti-rabies biologics for domestic animals\[9-11\].

Currently, killed rabies vaccines are often prepared from chicken embryo, BHK, or Vero cells and are available for human use and for pet animals. In China, Liaoning Chengda Animal Pharmaceutical Co.,Ltd. produced rabies vaccine by perfused culture of Vero cells with in 143 passages inoculated with PV2061 strain in bioreactor containing 25g/L of micro-carrier. The virus bulk was continuously harvested, then concentrated, inactivated and purified. The cell culture in bioreactor could be used for the large-scale production of rabies vaccine for human or animal use\[12\].

In this paper, we evaluated the safety, stability of the inactivated rabies vaccine PV2061 strain which was produced by Liaoning Chengda Animal Pharmaceutical Co.,Ltd. for its potential use as a vaccine against rabies in vivo. We evaluated its immunogenicity by examining humoral responses and tested the immune protection provided by the vaccine by using a lethal challenge assay in dog model.
Materials and Methods

1. Materials

1.1 Vaccines and virus

Inactivated rabies vaccine, PV2061 Strain (lot: 20061001, 20061002, 20061003, 20061004, 20061005, 20061006, 20061007, 20061008) which produced by Liaoning Chengda Animal Pharmaceutical Co., Ltd.商 品 化 可 用 RABV vaccine Rabisin (lot: L194957) purchased from CEVA and a freeze dried vaccine containing RABV attenuated vaccine (lot: 20061202) purchased from a company in China. The suckling-mouse-brain-adapted challenge virus, BD06 rabies virus strain and CVS-11 rabies virus strain was propagated and titrated in the brains of adult mice. The virus was harvested and then resuspended in PBS, stored at -80°C.

1.2. Dogs

14,000 dogs (The domestic dogs of different strains were from Guangxi province) were used. They were 4 weeks to 9 years old at the time of their injection. Animals were randomly assigned to four treatment groups of 10,000 dogs (group 1) and 4 control groups of 1000 dogs each group (group 2, 3, 4). The dogs in four groups were housed by their hosts separately.

2. Methods

2.1 Immunization

All vaccines were administered by the subcutaneous route. The dogs of group 1 treatment group were all vaccinated with PV2061 inactivated rabies vaccine but different doses, and this group was composed of 5 sub-groups: sub-group 1, sub-group 2, sub-group 3, sub-group 4 and sub-group 5.

In sub-group 1: 7,400 dogs were vaccinated with standard-dose PV2061 (1ml of PV2061 Inactivated Rabies Vaccine).

In sub-group 2: 1000 dogs were vaccinated with half of standard-dose PV2061.

In sub-group 3: 1000 dogs were vaccinated with a quarter of standard-dose PV2061.

In sub-group 4: 500 dogs were vaccinated with double standard-dose PV2061.

In sub-group 5: 100 dogs were vaccinated with tenfold standard-dose PV2061.

There were 1000 dogs in each control groups (group 2, 3, 4) that were inoculated...
with PBS, RABV vaccine Rabisin purchased from CEVA and freeze dried vaccine purchased from a company in China, respectively.

Infected animals were observed for 30 min for symptoms after rabies vaccines infection, and adverse reactions were also observed after 2 days, 5 days, 10 days, 14 days, 30 days and 120 days.

2.2 Immunofluorescence

Blood samples were collected from the jugular vein on dry tubes on Days 0, 7, 28, 90, 180, 365. Blood was also collected on 60 and 90 days post-challenge. In each group, 10% of dogs were collected blood. 691 samples were collected Sub-group 1. 95 samples were collected in Group 2. 98 samples were collected in Group 3. 100 samples were collected in Group 4.

Sera samples before immunity were assayed for antibodies by fluorescent antibody virus neutralization test (FAVN). Dogs with rabies antibodies titer higher than 0.21 IU/mL were considered as serologically positive for rabies. These dogs were deleted in this study. And the dogs whose antibody titer was lower than 0.1 IU/mL were available. In all of the available samples, 100 samples were selected randomly to rabies neutralizing antibody assay in sub-group 1 and 30 samples were selected in each of three control groups.

2.3 Rabies neutralizing antibody assay

For neutralization titer measurement, serum samples from the immunized dogs were serially diluted (from $10^{-1}$ to $10^{-3}$) with PBS and mixed with equal volume of CVS-11 rabies virus strain (100LD$_{50}$/0.03ml). The mixture was incubated at 37 °C for 1 h to neutralize the infectious virus and then the mixture was inject into the brains of adult mice for infection. After 4-day culture, the neutralization titers were read as the highest dilutions that could result in a 50% death. The antibody titers were log transformed (base 10) in order to normalize the distributions.

2.4 Rabies virus challenge

One year after the vaccination, thirty dogs that vaccinated with PV2061 and twenty control dogs whose rabies Ab titre were less than 0.21 IU/mL were selected randomly. It was decided to challenge a sufficient number of animals to ensure
compliance with the Chinese pharma-
copeia. For humane reasons, the
remaining dogs were not all
challenged. In the study, the control
dogs with the lower rabies antibody
titer were selected from Changchun
province. The dogs were challenged by
inoculation of 1 mL of rabies virus in
the neck muscles. The inoculums was a
homogenate of mouse brain received
$10^5$ mice LD50. The dogs were
examined daily for 90 days
post-challenge. When severe signs of
rabies infection were observed, animals
were anaesthetized, blood was
collected and the animal was
euthanized. Brain tissues were
collected at necropsy and tested by
immunofluorescence for Rabies. All
surviving animals were euthanized
after the 90-day follow up period.
Blood and brain tissue was collected
for serology and rabies antigen
detection by rabies neutralizing
antibody assay.

Results

1. Clinical manifestations in vaccinated
dogs

In our studies of vaccinated dogs,
the clinical sign were of body temperature
elevation, the papules and advomit in
some vaccinated dogs. We divided clinical
manifestations into general symptoms and
local symptoms (Tab.1). All three vaccines
did not induce any clinical sings including
fever or weight loss after vaccination for
long time in this research, but the dogs
which vaccinated with PV2061Strain and
Rabisin have less severe effect than
attenuated vaccine.

2. Immunogenicity

Vaccination induced a seroconversion
in almost all dogs. The rabies Ab titer
reached the highest point in 4 weeks after
vaccination. The higher rabies Ab titers of
subgroup 1 and group 3 last at least a year.
Ab titers of group 4 remained unstable and
rapidly decreased after the highest point in
4 weeks after vaccination. The mean Ab
titre was higher in subgroup 1 and Group
3, than in group 4 which was combined
with attenuated vaccine (Fig. 1 and Tab.2).
All the control animals remained
seronegative until the rabies challenge
(rabies Ab titre <0.1IU/ml).

3. Efficacy

The non-vaccinated animals began
showing clinical signs of rabies approxim-
-ately 15 days following rabies infection (14.5 days on average, ranging from 11 to 19 days). They were euthanized following the onset of signs of rabies. All were found positive by immunofluorescence. In vaccinated animals, morbidity or mortality was observed in 4 animals and the dogs were found positive by immunofluorescence at the end of the study. Others (87.5%) were all found negative by immunofluorescence and those dogs were all survived after challenge (Tab.3).

<table>
<thead>
<tr>
<th>group</th>
<th>quantity</th>
<th>Temperature</th>
<th>Allergy</th>
<th>Vomit</th>
<th>Red</th>
<th>Swell</th>
<th>rhabdion</th>
<th>Total</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
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<td>7501</td>
<td>6</td>
<td>-</td>
<td>2</td>
<td>18</td>
<td>19</td>
<td>70</td>
<td>115</td>
<td>1.52</td>
</tr>
<tr>
<td>sub-group 2</td>
<td>1000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>2</td>
<td>0.20</td>
</tr>
<tr>
<td>sub-group 3</td>
<td>1000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>sub-group 4</td>
<td>500</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>6</td>
<td>4</td>
<td>9</td>
<td>24</td>
<td>4.80</td>
</tr>
<tr>
<td>sub-group 5</td>
<td>100</td>
<td>2</td>
<td>-</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>10</td>
<td>10.00</td>
</tr>
<tr>
<td>Group 3</td>
<td>1001</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>9</td>
<td>15</td>
<td>1.50</td>
</tr>
<tr>
<td>Group 4</td>
<td>1000</td>
<td>12</td>
<td>3</td>
<td>1</td>
<td>18</td>
<td>8</td>
<td>5</td>
<td>47</td>
<td>4.7</td>
</tr>
</tbody>
</table>

Figure 1. The rate of seroconversion to rabies. Sera were analyzed from day 0 (immunization), day 7 post immunization, day 28 post immunization, month 3 post immunization, month 6 post immunization and month 12 post immunization. Serum samples were tittered by FAVN.
<table>
<thead>
<tr>
<th>Days after vaccination</th>
<th>Rabies Antibody titer (IU/ml)</th>
<th>Total</th>
<th>Positive rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1</td>
<td>0.11-0.49</td>
<td>0.5-1.4</td>
</tr>
<tr>
<td>0 day</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7 days</td>
<td>10</td>
<td>69</td>
<td>15</td>
</tr>
<tr>
<td>28 days</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>3 months</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6 months</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12 months</td>
<td>-</td>
<td>-</td>
<td>15</td>
</tr>
</tbody>
</table>

**Discussion**

Rabies is present worldwide, and is still responsible for more than 55,000 human deaths every year, mainly in Asia and Africa [13, 14]. It is endemic in bats and several other species (fox, raccoon-dog, raccoon, skunk etc.) forming the main reservoir of infection for dogs. Globally, the dog is the primary source of infection for humans [15, 16]. Currently, dogs are not vaccinated sufficiently, and potentially contributing to the high number of rabies cases in some regions. Several marketed vaccines, mostly adjuvant inactivated vaccines have been shown to protect dogs 1 to 4 years depending on the manufacturers [17-19].

In this research, we compared the immunogenicity of three vaccines in dogs. All three vaccines did not induce any clinical signs including fever or weight loss after vaccination for a long time in this research. But the dogs which vaccinated with PV2061 Strain and Rabisin have less severe clinical manifestations than attenuated vaccine. The protection of immunized animals was largely dependent on the induced humoral immune response. The mean Ab titer was higher in subgroup 1 and Group 3, than in group 4 that was combined with attenuated vaccine. What is more, Ab titer last longer time in subgroup 1 and Group 3, at least for a year.

To evaluate the protection effectiveness of PV2061 strain, an challenge experiment was carried out. In this experiment, the 30
Table 3: The condition of vaccinated dogs after challenge

<table>
<thead>
<tr>
<th>Number of dogs</th>
<th>Before challenge</th>
<th>60 days after challenge</th>
<th>90 days after challenge</th>
<th>Survive or Die</th>
<th>The result of immunofluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.17</td>
<td>0.87</td>
<td>0.51</td>
<td>Survive</td>
<td>negative</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>Died after 10 days</td>
<td>positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5.92</td>
<td>92.32</td>
<td>7.79</td>
<td>Survive</td>
<td>negative</td>
</tr>
<tr>
<td>4</td>
<td>5.92</td>
<td>2.60</td>
<td>0.87</td>
<td>Survive</td>
<td>negative</td>
</tr>
<tr>
<td>5</td>
<td>1.97</td>
<td>3.42</td>
<td>1.14</td>
<td>Survive</td>
<td>negative</td>
</tr>
<tr>
<td>6</td>
<td>4.50</td>
<td>17.77</td>
<td>10.26</td>
<td>Survive</td>
<td>negative</td>
</tr>
<tr>
<td>7</td>
<td>0.29</td>
<td>7.79</td>
<td>2.60</td>
<td>Survive</td>
<td>negative</td>
</tr>
<tr>
<td>8</td>
<td>0.50</td>
<td>0.87</td>
<td>0.39</td>
<td>Survive</td>
<td>negative</td>
</tr>
<tr>
<td>9</td>
<td>10.26</td>
<td>30.77</td>
<td>5.92</td>
<td>Survive</td>
<td>negative</td>
</tr>
<tr>
<td>10</td>
<td>0.50</td>
<td>5.92</td>
<td>2.60</td>
<td>Survive</td>
<td>negative</td>
</tr>
<tr>
<td>11</td>
<td>0.50</td>
<td>3.42</td>
<td>0.50</td>
<td>Survive</td>
<td>negative</td>
</tr>
<tr>
<td>12</td>
<td>5.92</td>
<td>4.50</td>
<td>1.14</td>
<td>Survive</td>
<td>negative</td>
</tr>
<tr>
<td>13</td>
<td>13.50</td>
<td>10.26</td>
<td>1.14</td>
<td>Survive</td>
<td>negative</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>Died after 12 days</td>
<td>positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>7.79</td>
<td>10.26</td>
<td>0.50</td>
<td>Survive</td>
<td>negative</td>
</tr>
<tr>
<td>16</td>
<td>17.77</td>
<td>30.77</td>
<td>5.92</td>
<td>Survive</td>
<td>negative</td>
</tr>
<tr>
<td>17</td>
<td>0.45</td>
<td>Died after 12 days</td>
<td>positive</td>
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</tr>
<tr>
<td>18</td>
<td>7.79</td>
<td>30.77</td>
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<td>Survive</td>
<td>negative</td>
</tr>
<tr>
<td>19</td>
<td>1.97</td>
<td>4.50</td>
<td>3.42</td>
<td>Survive</td>
<td>negative</td>
</tr>
<tr>
<td>20</td>
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<td>2.26</td>
<td>0.55</td>
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<td>negative</td>
</tr>
<tr>
<td>21</td>
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<td>positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>1.97</td>
<td>1.97</td>
<td>1.50</td>
<td>Survive</td>
<td>negative</td>
</tr>
<tr>
<td>23</td>
<td>0.5</td>
<td>0.70</td>
<td>0.50</td>
<td>Survive</td>
<td>negative</td>
</tr>
<tr>
<td>24</td>
<td>1.14</td>
<td>13.38</td>
<td>1.29</td>
<td>Survive</td>
<td>negative</td>
</tr>
<tr>
<td>25</td>
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<td>10.26</td>
<td>0.78</td>
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</tr>
<tr>
<td>26</td>
<td>0.22</td>
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<td>Survive</td>
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<tr>
<td>27</td>
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</tr>
<tr>
<td>28</td>
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<td>2.60</td>
<td>Survive</td>
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</tr>
<tr>
<td>29</td>
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<td>0.66</td>
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</tr>
<tr>
<td>30</td>
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<td>1.14</td>
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<td>Survive</td>
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</tr>
<tr>
<td>31</td>
<td>1.97</td>
<td>5.92</td>
<td>0.45</td>
<td>Survive</td>
<td>negative</td>
</tr>
</tbody>
</table>

dogs vaccinated PV2061 strain and 20 control dogs were selected randomly. Results showed that most of vaccinated dogs’ sera was seroconverted and developed a high antibody response to rabies virus except 3 dogs before challenge. And the dogs that were seroconverted were completely protected against rabies virus challenge. The dogs in the control group didn’t develop antibody response.
response, and none of the dogs were survived after challenge. The protection rate is 87.5%.

In summary, the present study show that the inactivated rabies vaccine PV2061 strain can protect dogs from challenge. The results suggest that the PV2061 strain strain is an effective vaccine.

References

