Development of a New Inactivated Vaccine for Equine Rhinopneumonitis Virus in Japan

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In early 1967, an outbreak of abortion and stillbirth caused by Equine Rhinopneumonitis Virus (ERV) occurred mainly with imported horses in the Hidaka district of Hokkaido. Kawakami et al.[3] found out that the virus HH-1 strain isolated from the aborted fetus was different in antigenicity from Japanese native strains (TH-20[2] and H-45[7]) so far known, showing the same antigenicity of the virus which is regarded to cause abortions in the United States of America. Since that outbreak of abortion caused by ERV, it has been keenly desired to establish preventive measures.

Shimizu et al.[6,8] reported the result of preliminary experiment with colts that the pre-infection either of native strain or attenuated virus of KyB strain (isolated from aborted fetus in U.S.A. and adapted to hamster[1]) was effective in preventing intranasal challenge of HH-1, and that the LKK method, in which inactivated vaccine of KyD strain (akin to KyB[1]) or that of the native strain were inoculated twice, after inoculating the native live virus twice, was able to protect intratracheal infection of HH-1 strain[9]. Furthermore, in view of the fact that in Japan almost all horses reaching the age of pregnancy are considered to have experienced native virus infection, they[7] suggested the possibility of preventing abortion only by the use of inactivated vaccine. Kawakami et al.[5] applied the Shimizu's method experimentally to pregnant horses, and reported that the inoculation of live (a native strain) and inactivated (KyD strain) vaccine is able to prevent abortion and stillbirth.

Now that the horses in Japan, especially pregnant horses, have mostly experienced the native virus infection as pointed out by Shimizu et al.[7], an attempt was made to examine the immunity effect of inactivated vaccine alone without live virus inoculation.

At first, the culture method of virus was established by propagating the HH-1 strain in calf kidney cell line (CKT) after an adaptation to bovine kidney cell culture. Next, the method of mass-culture was established by using the suspension cell culture method, as the CKT cells can be cultured in big suspension cell culture tanks. Then, the production of inactivated vaccine having higher titer values was made possible by partial purification after concentration of this virus. The vaccine was proved to be safe to horses and highly precautionary against abortion.

Preparation of seed virus strain and production of inactivated vaccine

1) Seed virus strain for vaccine production

The HH-1 strain, isolated from naturally occurred aborted fetus through primary horse kidney cell culture, was further cultured for 3 serial passages. Then, it was cultured by

primary bovine kidney cell culture up to the 78th serial passage, followed by 10 serial passages in calf kidney cell line (CKT), and finally it was used as the seed virus for vaccine production.

2) Cultivation of cells and propagation of virus

To determine the optimum condition for the suspension culture system of CKT cells, the relation between incubation temperatures and seed virus propagation was examined at 30°, 33°, 35°, and 37°C at the cell concentration of 1.3×10^6 cells/m*l* and at the amount of inoculum, m.o.i (multiplicity of infection)= 0.1. At 37°C, the virus reached 10^7 $TCID_{50}$/m*l* after 16 hrs, and the maximum titer, $10^{7.75}$, after 40 hrs. At 35°C, the virus propagation tended to delay as compared to 37°C, but reached $10^{8.0}$ after 52 hrs. This was followed by 33°C and then 30°C. Thus, 37°C was known to give the most stable growth curve, and hence taken as the most suitable incubation temperature.

Next, the relation between m.o.i. and virus yields was examined with varying m.o.i., 20, 2, 0.2, and 0.02, at the cell concentration of 1.5×10^6 cells/m*l* at 37°C. At any m.o.i., the virus titer reached the highest after 35 hrs, but the growth curve was most superior at m.o.i.=2.

Then, the seed virus propagation at the scale of 50 *l* suspension culture tank was attempted aiming at the practical vaccine production. The CKT cells in 50 *l* tanks were cultured at 37°C for 2-3 days. When they reached 1.5×10^6 cells/m*l*, they were chilled to 4°C and precipitated. The sediment was collected by centrifuging and suspended at the concentration of about 3.0×10^7 cells/m*l*. After the virus was inoculated at m.o.i.=2, fresh medium was added to adjust the cell concentration to 1.5×10^6 cells/m*l*, and incubated at 37°C. As shown in Fig. 1, the virus titer reached $10^{8.5}$/m*l* after 20 hrs, showing a tendency of further gradual increase with time. After 30 hrs, supernatant of centrifugation was collected to give the virus solution. The virus titer was usually $10^{8.5}$/m*l*, with protein

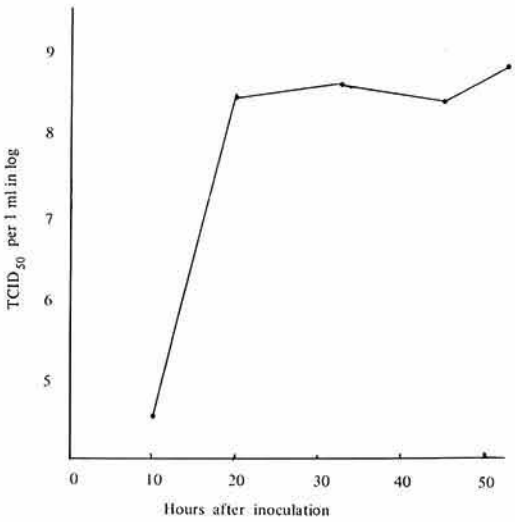

Fig. 1. Growth curve of the seed virus strain in 50 liter CKT cell suspension culturs
Cell concentration = 1.5×10^6/m*l*
Virus inoculum = m. o. i. 2
Incubation temprature = 37°C

concentration of 3.2 mg/m*l*.

3) Concentration and partial purification

After adding polyethylene glycol #6000 at the rate of 10%, the virus solution was stirred overnight at 4°C, and submitted to continuous centrifugation at 9,000 r.p.m. and flow rate less than 3 *l*/hr. The sediment, after adding phosphate buffered saline (PBS) of pH 7.2 at the rate of 1/40 volume of the sediment, was resuspended by ultrasonic treatment and centrifuged for 30 min at 5,000 r.p.m. to obtain supernatant as virus solution. By this procedure, the virus titer was not increased, but protein which is regraded to cause side effects was reduced to about 1/8 of the initial quantity: the virus solution showed the virus titer of $10^{8.5}$/m*l* and protein content of 0.4 mg/m*l*. Thus, the partially purified virus was obtained.

4) Method of inactivation and adjuvant

Formalin was added to the concentrated virus to give the final concentration of formalin of 0.2%, and the mixture was kept at 4°C. After 20 hrs of this treatment infectivity of

the virus disappeared completely, but for the sake of safety, a 48 hrs treatment was made for producing inactivated virus. Various kinds of adjuvant were prepared, and tested by actual inoculation to horses. As a result, aluminum phosphate gel was found most superior in safety and potency.

Safety and potency of inactivated vaccine

1) Safety test

Safety of the inactivated vaccine prepared was examined using guinea pigs, mice, and horses. To a guinea pig 2 m*l* of the inactivated vaccine was injected intramuscularly to the rump, 1 m*l* each to its left and right side. To a mouse, 0.5 m*l* was injected to the abdominal subcutaneous region. Swelling and induration of inoculated sites and other general symptoms like hair, anorexia, body weight, etc, were compared with non-injected control groups. The treated guinea pigs showed no clinical abnormalities at all, with the body weight gain same as the controls. Some of the treated mice showed swelling and induration at the inoculated sites, but no other clinical symptoms were observed, with the same body weight gain as the controls.

Using 10 horses, 5 m*l* (1 dose) each of the vaccine was inoculated intrasubcutaneously to their neck two times with an interval of 4 weeks. No clinical symptoms like pyrexia and others were recognized at all. Furthermore, 10 m*l* (2 doses) each of the vaccine was inoculated two times with an interval of 5 weeks to 7 horses. No abnormalities like pyrexia and others was observed, except slight swelling in the neck subcutaneous region occurred after the second inoculation with 4 out of 7 horses, but it disappeared after about 1 month.

2) Protection test in hamster

Hamsters inoculated with the vaccine are resistant against the attack of KyD strain virulent to hamsters[1, 4]. As the 50% effective dose (ED_{50}) of the inactivated vaccine shown with hamster is correlated to the antibody production of horses to a certain extent, it is used for potency tests of the vaccine. The inactivated vaccine was diluted to 1:250–1:4000 in PBS with two-fold dilution levels, and inoculated intraperitoneously to hamsters of 6 weeks of age at a dose of 1 m*l*. After 3 weeks, KyD strain with LD_{50} of 10^4 was inoculated intraperitoneously to the hamsters and ED_{50} was calcuated after a 1 week observation. Table 1 shows the relationship between the virus titer prior to the inactivation and ED_{50} shown with hamsters. For the virus titer of $10^{9.125}$/m*l*, the ED_{50} was 1:2000, and for $10^{8.75\text{-}9.0}$/m*l*, it was 1:1000.

Table 1. Protection test* of inactivated vaccine in hamster, showing the relationship between virus titer and protective titer in hamster

Virus titer before inactivation (log $TCID_{50}$/m*l*)	Dilution of vaccine in PBS				ED_{50}
	1:500	1:1000	1:2000	1:4000	
9.125	4/4**	4/4	2/4	0/4	1:2000
9.0	4/4	2/4	0/4	0/4	1:1000
8.75	4/4	1/4	1/4	0/4	1:1000

* Six old hamsters, vaccinated intraperitoneously with 1 m*l* of variously diluted vaccines, were inoculated through the same route with 1 m*l* of KyD strain (10^4 MLD) after 3 weeks. The hamsters were observed for 1 week after the challenge.
** No. of survivals/ No. of vaccinated.

Table 2 shows relationship between ED_{50} shown by hamsters and neutralizing antibody-titer shown by horses inoculated with the inactivated vaccine. By diluting the vaccine with PBS, the vaccines with ED_{50} of 1:750, 1:500 1:250 or 1:125 were prepared, and each of them was inoculated two times at the interval of 4 weeks to 5 horses. Antibody titers of pre-immune sera of each horse were $1: <1\sim2$. With the horses inoculated with the vaccine of ED_{50} of 1:750, significant increase of neutralizing antibody titer was observed in 4 horses (80%) out of 5. Similarly, the significant increase was recognized in 60% horses for 1:500 and 1:250, and only in 10% for 1:125.

Table 2. Relationship between antibody-titer and 50% effective dose (ED_{50}) observed in experimental immunization of horses with inactivated ERV vaccine

ED_{50}	No. of horses	Neutralizing antibody-titer in sera		No. of animals with significant increase of antibody/No. of vaccinated animals (%)
		pre-immune	1 month post-immune	
1 : 750	1	<1	4*	80
	2	2	8*	
	3	<1	8*	
	4	1	8*	
	5	2	4	
1 : 500	6	<1	8*	60
	7	1	2	
	8	2	8*	
	9	1	4*	
	10	2	4	
1 : 250	11	1	2	60
	12	<1	8*	
	13	1	4*	
	14	2	4	
	15	2	8*	
1 : 125	16	2	4	20
	17	<2	2	
	18	<2	2	
	19	<2	2	
	20	<2	8*	

* Significant increase of antibody-titer

Thus, it was made clear that the antibody-titer of vaccinated horses can be predicted to a certain extent from ED_{50} in hamsters, and hence the latter can be used as an index in determining vaccine effectiveness.

Application of ERV vaccine to pregnant horses

1) Experiment on vaccination

Result of application of ERV vaccine to pregnant horses with an aim of preventing abortion and stillbirth is shown in Table 3. Ten pregnant horses were divided into an immune group and a control group, each consisted of 5 horses. To the former, 2 m*l* of live virus, TH-20 strain, was inoculated intranasally and 10 m*l* of the inactivated vaccine was inoculated intramuscularly to the neck after 4 and 10 weeks, respectively. At 2 weeks after the second vaccination, both groups were challenged by the highly virulent HH-1 strain. Before the challenge, the horses were of 247–299 days of pregnancy and neutralizing antibody-titer was 1:8~32 for the immune group while 1: <1 for the control group. After the challenge, the immune group showed no abnormality: the virus was not detected in nasal swab and normal birth occurred in all cases. On the contrary, in the control group pyrexia, nasal discharge, swelling of mandibular lymphnodes and leukopenia were observed, and abortion and stillbirth occurred with 3 out of 5 horses after 16, 26, and 57 days, respectively. The virus was recovered from the aborted fetus in the control group, but not recovered from either mother horses or colts in the immune group.

Thus, the effectiveness of the inactivated

Table 3. Experiment on application of ERV vaccine to pregnant horses

Group	No. of horses	Pre-immune		Pre-challenge		Findings after challenge					Results			
		Antibody titer	Days of pregnancy	Antibody titer	Days of pregnancy	Pyrexia (Max.)	Nasal dischage	Swelling of lymph.*	Leukopenia	Recovery of virus by nasal swab	Foaling	Recovery of virus: Fetuses	Recovery of virus: Mares	No. of abortion and stillbirth / No. of challenge-exposed horses
Immune group	1	<1	192	16	282	−	−	−	−	−	Normal	−	−	0/5
	2	<1	157	16	247	−	−	−	−	−	Normal	−	−	
	3	<1	209	32	299	−	−	−	−	−	Normal	−	−	
	4	<1	197	8	287	−	−	−	−	−	Normal	−	−	
	5	1	165	32	255	−	−	−	−	−	Normal	−	−	
Control group	6	<1	197	<1	287	+ (38.9)	+	+	+	+	Normal	−	−	3/5
	7	<1	179	<1	269	+ (39.1)	+	+	+	+	Abortion (16 days)	+	−	
	8	<1	209	<1	299	+ (38.9)	+	+	+	+	Normal	−	−	
	9	<1	201	<1	291	−	+	+	+	+	Abortion (26 days)	+	−	
	10	<1	169	<1	259	+ (39.1)	+	+	+	+	Stillbirth (57 days)	+	−	

The immune group was inoculated intranasally with 2 m*l* of the live TH-20 strain of EVR of 10^3TCID$_{50}$/m*l*, and then vaccinated intramuscularly 2 times with 10 m*l* of the inactivated vaccine after 4 weeks and 10 weeks. Both groups were challenge-exposed intranasally with the HH-1 strain of ERV (10^4MLD).
* lymph.: Mandibular lymphnodes.

vaccine was confirmed. For successful prevention of abortion and stillbirth, it is considered that the serum antibody-titer of pregnant horses must be kept higher than 1 : 8.

2) Field application test

As shown by the result of the above experiment, it is necessary to maintain serum antibody-titer of pregnant horses higher than 1:8 for preventing abortion and stillbirth caused by ERV, so that the inactivated vaccine was inoculated two times at the interval of 6 weeks to 7 pregnant horses, and production and persistence of the antibody were observed over a period of 13 weeks. Each inoculation was made intrasubcutaneously to a neck with 10 m*l* of the vaccine showing ED$_{50}$ of 1:250~1,000 in hamster. The result is shown in Fig. 2. The serum antibody-titer before the vaccination was 1:2 in 6 horses and 1:1 in 1 horse. At 1 week after the first vaccination, the antibody-titer began to increase, and reached the highest level, 1:4~16, after 2–3 weeks. After that, 6 out of the 7 horses maintained 1:4~8 until the second vaccination, and 1:8~16 until 10–11th week after the first vaccination.

Antibody-titers of pregnant horses after the vaccination are shown in relation to the titers before the vaccination in Table 4, which indicates that the lower the antibody-titer of pre-immune sera the higher is the rate of increase of the titer after the vaccination. Horses which had the antibody titer of pre-immune sera lower than 1:8 showed the mean antibody-titer of post-immune sera of 1:7.2~12.8. On the other hand, the horses showed the antibody-titer of pre-immune sera higher than 1:16 gave the mean titer of 1:14.6. As the antibody-titer effective in preventing abortion and stillbirth is regarded to be higher than 1:8, the use of this inactivated vaccine is possible to prevent abortion and stillbirth caused by ERV.

It was made clear from the surveys so far done that the abortion and stillbirth caused by

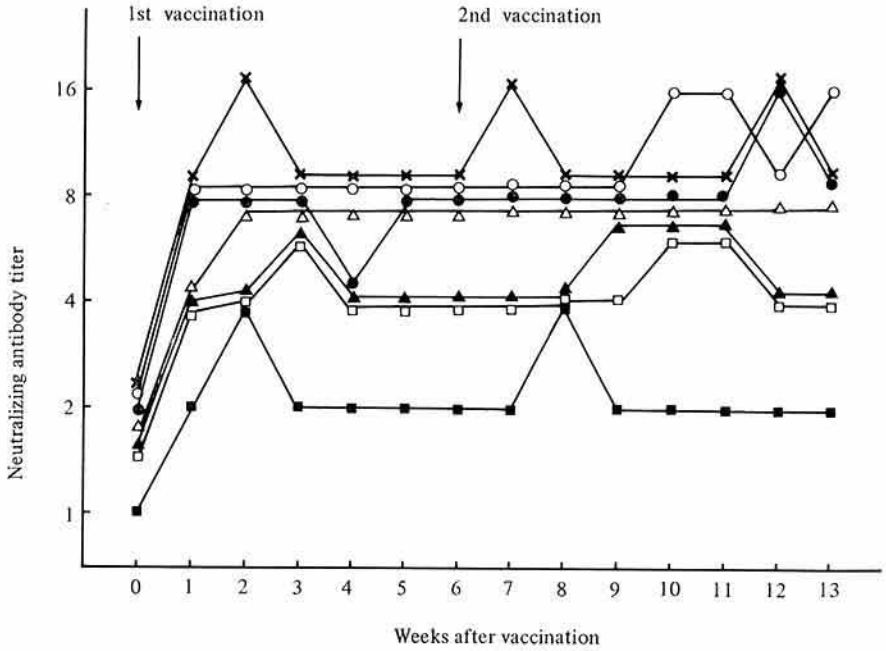

Fig. 2. Production and persistence of serum antibody after vaccination.
Seven horses were vaccinated intrasubcutaneously with 10 m*l* of ERV vaccine (1:250 to 1000 ED_{50}).

Table 4. Neutralizing antibody-titer of the vaccinated horses

Neutralizing antibody titer of pre-immune sera	<1	1	2	4	8	≧16
Mean of Neutralizing antibody titer of post-immune sera	12.8	7.8	8.3	7.2	10.6	14.6
95% confidence limits of mean antibody titer	±0.50	±0.51	±0.37	±0.40	±0.62	±0.60
Standard deviation	1.14	0.89	0.94	0.97	0.70	0.96
Number of animals	19	14	27	25	7	12

ERV occur frequently at the third trimester[3], so that the timing of the vaccination (repeated two times) focused on this stage will be very effective.

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