STUDIES ON TRIPLEX VACCINE AGAINST RABBIT HAEMORRHAGIC DISEASE, *BORDETELLA BRONCHISEPTICA* DISEASE AND PASTEURELLOSIS

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Abstract - Rabbit haemorrhagic disease (RHD), *Bordetella bronchiseptica* (Bb) disease and *Pasteurella multocida* (Pm) disease are three main infectious diseases in rabbits. The number of Bb and Pm cultivated by the new method of liquid cultivation developed in our laboratory reached 1.86-2.3 x 10^{10} and 2-2.6 x 10^{10} living cells/ml, respectively. Every rabbit in immunised groups was inoculated subcutaneously with a dose of 1 ml triplex vaccine prepared with RHD, Bb and Pm. The immune protective rate against RHD was 100% (28/28) 5 days after vaccination and 6.5 months later the efficiency of immunity remains 100% (23/23). The average rates of protection against Bb and Pm 10-14 after vaccination were 88.9% (24/27) and 88.5% (23/26), and were 72. 7% (8/11) and 75.0 (9/12) 6.5 months later. The triplex vaccine was stored more than 6 months in refrigerator (4-8°C) or 4 to 6 month at room temperature (<25°C) without reduction of efficiency. Dynamics of the antibody were consistent with results of the challenge. The results from vaccination of more than 295 000 rabbits in the field showed the good safety and the efficacy of the vaccine.

INTRODUCTION

Rabbit haemorrhagic disease (RHD), *Bordetella bronchiseptica* (Bb) and *Pasteurella multocida* (Pm) are three main infectious diseases with broad prevalence, high morbidity and mortality in rabbits (DU NIANXIN *et al.*, 1988; AL-LEBBAN *et al.*, 1988; TONG CHENGGANG *et al.*, 1994). The three single vaccines against the three diseases have obvious efficacy, but three inoculations are needed for a rabbit, which spent much time and cost (WANG YONG-KUN *et al.*, 1984; TONG CHENGGANG *et al.*, 1986). Development of triplex vaccine against RHD, Bb and Pm will overcome these defects.

MATERIAL AND METHODS

Strains of virus and bacteria

RHDV-TRH was isolated from the liver of a dead rabbit in Zhijiang, China. Rabbits 1.5-2kg body weight subcutaneously inoculated with 1 ml of 10^{-1} saline diluted liver virus tissues died above 80% within the 24-72 hrs post-infection ,with typical lesions of RHD. The haemagglutination titre of 1% human "o" type red blood cells by RHDV was >2560.

Bordetella bronchiseptica R24, TR105 were type I isolated from dead or rhinitis rabbits. Healthy rabbits inoculated in chest with 0.5 ml bacteria suspension from one brood agar slant washed with 3 ml martin broth died or had severe pathological change.

Pasteurella multocida C51-2, C51-3, RP1211 were type A. C51-2 and C51-3 were supplied by National Institute for the Control of Pharmaceutical and Biological Products. RP1211 was isolated from dead rabbit in Zhijiang, China. Rabbits challenged with 2-6 living organisms died within 72 hrs.

Preparation of triplex vaccine

RHD vaccine : Liver and spleen from rabbits infected with RHDV were homogenised. This vaccine was prepared according to the code issued from Bureau of Animal Industry, Ministry of Agriculture of China.

Bb vaccine : Typical colonies of the organisms growing on sheep blood Martin Broth agar plate were selected and inoculated to Martin Broth served as seed culture. The medium for the bacteria growth was Martin Broth containing 0,1% splitting sheep blood etc... The culture of the bacteria was made at 37°C for 18-22 hrs with aeration with filtered air by modified liquid culture technology. The organisms usually grow up to 1.83-2 x 10^{10} living cells/ml at least and sometimes up to about 4 x 10^{10} cells/ml. The bacteria were inactivated with 0.3% Formalin at 37°C for 24 hrs.

Pm vaccine-The preparation method was the same as Bb's, but the temperature for cultivation was 38°C. The organisms usually grow up to 2-2.6 x 10^{10} living cells/ml and could reached 4.07 x 10^{10} cells/ml.
Triplex vaccine The three single vaccines were mixed in 3 identical parts into a triplex vaccine. A dose of the triplex vaccine contained liver and spleen tissues with RHDV 0.05 mg , Bb 6 x 10^9 CFU and Pm 6 x 10^9 CFU about.

Examinations of triplex vaccine

Sterility test - 0.2 ml of vaccine were inoculated into duplicate martin broth, anaerobic cooked meat and live broth, blood agar and martin agar slants. No bacteria growth occurred after 5-7 days incubation.
Safety test - Four normal susceptible rabbits for a batch were injected subcutaneously with 5 ml vaccine per-rabbit. All of them were healthy within 7 days.

Animals and methods for immune test
Animal s- Healthy young rabbits with body weight 1.5-2.5 kg were used. Before test all of the rabbits were negative from Bb ant Pm and serum antibody titres against RHDV were 0-1:4 by HI test.
Challenges -Every tested rabbit was subcutaneously injected 1 ml of 1/10 diluted livers tissues with RHDV. As to Bb, rabbit was injected with 1/6 agar slant bacteria in chest. As to Pm, every animal was challenged with 2 MLD (1 MLD was 1-3 living organisms).
Detection of antibody was made by RI and dot-ELISA test.

RESULTS

Potency of immunity

76 susceptible normal rabbits were applied for test of five batches. Every animal was subcutaneously injected with 1 or 2 ml triplex vaccine. Five to seven days later, 28 rabbits were challenged with RHDV, 10-14 days later, 39 and 37 rabbits were challenged with Bb and Pm, respectively. The results are shown in Table 1.

Table 1 : Determination of potency of immunity

Dose (ml)	No. Survivors / total no. RHD		Bb		Pm	
	IG*	CΔ	IG	C	IG	C
1	28/28	1/10	24/27	1/20	2326	0/20
2	/	/	11/12	1/10	10/11	0/10

*IG = Immunized group ΔC = Control

Results in Table 1 indicate that the immune protection rates against RHD, Bb and Pm were 100%, 88.5% and 88.3i in 1 ml group, and 100%, 91.7% and 90.9% in 2 ml group, respectively. By t-test, there was no obvious difference between them. So a practical dose would be 1 ml.

Period of immunity produced

56 susceptible normal rabbits were immunised with the triplet vaccine from three batches. The rabbits were challenged on the 3rd, 5th, 7th, 10th, 14th days post-vaccination. The results art shown in Table 2

Table 2 : Determination of period of immunity produced

Days	No. Survivors / total no. RHD		Bb		Pm	
	IG	C	IG	C	IG	C
3	5/12	0/10	/	/		/
5	12/12	0/8		/		/
7	20/20	1/10	7/10	0/4	8/10	0/5
10	/	/	8/11	1/4	10/12	0/4
14	/	/	9/10	0/4	11/12	0/4

The rates of immune protection against RHD were 41.7%, 100% and 100% on the 3rd, 5th and 7th day post-immunisation. The rates of immunity against Bb and against Pm were 70.0%, 72.7%, 90.0% and 80.0%, 83.3%, 91.7% respectively on the 7th, 10th, 14th day post-immunisation. In general, high immunity could be produced against RHD, Bb and Pm on the 5th, 10th and 10th day post-vaccination.

Duration of immunity

47 susceptible rabbits vaccinated with 3 batches of the vaccine were challenged 4.5 or 6.5 months later. The results are showed in Table 3.

Table 3 : Determination of durations of immunity of triplex vaccine

Months after immunization	No. Survivors / total no.					
	RHD		Bb		Pm	
	IG	C	IG	C	IG	C
4.5	24/24	0/4	11/12	0.3	10/12	1/4
6.5	23/23	1/4	8/11	0/4	9/12	0/4

Table 3 showed that the protective rate of triplex vaccine was 100% against RHD after 6.5 months. After 4.5 and 6.5 months, the rates of protection were 91.7% and 72.7% against Bb, and 83.3% and 75.0% against Pm.

Stability of triplex vaccine in storage

50 susceptible rabbits were vaccinated with 3 batches of the vaccines that were stored for 121-196 days at room temperature (through the winter) or stored for 198-237 days in a refrigerator (4-8°C). The results of challenge are shown in Table 4.

Table 4 : The triplex vaccine effect after storage

Methods and days of storage	No. Survivors / total no.					
	RHD		Bb		Pm	
	IG	C	IG	C	IG	C
Room temperature 121-196 days	26/26	1/8	12/14	0/8	9/12	1/8
Rifrigerator 198-237 days	24/24	0/4	10/12	0/4	10/12	0/4

After storage for 237 days in refrigerator (4°C) and for 196 days at room temperature (<25°C) the triplex vaccines could still provide a reasonable protection.

Dynamics of antibodies in rabbits after vaccination

Antibodies of 7 rabbit sera were determined at intervals after vaccination. Antibodies against RHDV were detected by HI and that against Bb by dot-ELISA. The results are shown in Table 5.

Table 5 : Dynamics of antibody of rabbitss of vaccination with triplex vaccine

Days	P	3	7	15	22	29	36	45	60	75	90	108	120	136	150	165	180
Rabbits	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
RHD HI Titre (log^2)	0	0	5.6	8.1	8.8	8.3	8.3	8	7.7	7.5	7.5	7	6.7	6.3	6.3	6.3	6.2
Bb (Dot-ELISA)	0	0	400	1664	3328	4352	1664	1620	1600	1280	1280	840	560	360	280	150	140

P = pre-immunization

Antibodies against RHDV - The titres were 0 and 25.6 on the 3rd and 7th day post-immunisation, and rose obviously on the 15th day. The highest (average HI $2^{8.8}$) was observed on the 22nd day. The high level ($2^{6.2}$) of titres did not drop until 180 days.
Antibody against Bb-The titres were 0 and 1/400 on 3rd and 7th day, and rose notably on 15th day and reached the highest (1/4352) on the 29th day. It remained 1/140 on the 180th day post-immunisation.

Application in field

From 1992 to 1994, more than 295 000 rabbits were vaccinated with 8 batches of triplex vaccine in Shengxian county. No harmful reactions were found post-vaccination. Within 6 months after vaccination there were no RHD and acute pasteurellosis happening in all immunised rabbits. The morbidity and mortality of infectious rhinitis of rabbits decreased significantly.

DISCUSSION

1. By the modified liquid culture technology, the organism content of Bb and Pm per-millilitre culture increased 2-3 times than the reported methods (YU GUANGHAI *et al*, 1990; DONG YAFANG *et al.*, 1991) . In this way the production of the vaccine and the efficiency of immunity could be improved and the cost of the production reduced.
2. Experimental results showed the rabbits immunised could produce strong immunity against RHD in 5 days and against Bb and Pm in 10 days post-immunisation. After 6.5 months, the protective rates were still 74-100%. So the immune efficacy of triplex vaccine was reliable.
3. Bb and Pm are both Gram-negative bacteria that have the lipopolysaccharide (LPS) of adjuvant activity(WANG CUILAN et al., 1990).After three single vaccines were mixed, the LPS of one bacterium serves as an adjuvant for another. Interferon (IFN) induced by RHDV could increase immune efficacy against the two bacteria.
4. It was reported that the rabbits can be protected 24 hrs post-immunisation. The reason for it was that RHD vaccine could strongly induce the production of IFN. The titre of IFN started to rise as early as 6 hrs after inoculation. The highest was at 18 hrs. It begun to decrease at 30 hrs and on the 3rd day, the IFN decreased to lower level (LI SHENNIAN, 1988), but the antibody against RHDV were not produced. This could explain the lower protective rate (41.7%) against RHD at that time after vaccination of the triplex vaccine.
5. According to determination of antibodies, dynamics of antibodies against RHDV, Bb were consistent with the results of challenge. Dot-ELISA for determination of antibodies against Pm is establishing.
6. The results of the experiment and the application in field proved the high safety and immune efficacy of the triplex vaccine. It has the advantages of small dose (1 ml/rabbit), low cost and convenient conditions for storage and transportation. Compared with the single vaccines it could save 2/3 the expenses of vaccination.

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