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STUDIES ON EQUINE PARATYPHOID VACCINE
III. CLINICAL REACTIONS AND ANTIBODY RESPONSES
OF HORSES TO CHROME VACCINE OF
SALMONELLA ABORTUS EQUI

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Many studies on the improvement of equine paratyphoid vaccine have been performed. In a previous paper of this series the authors (1953) reported that the increase of immunizing dose of killed vaccine would make it possible to protect mice from infection with a large challenge dose of abortion bacilli, and also that the chrome vaccine prepared by ANDO's method (1951) was the most favourable in respect to its small toxicity. Moreover, TOBA and his associates (1952) reported on the increased antigenicity and reduced toxicity in animals of vaccine emulsified in vegetable oil which they called "lipovaccine". This vaccine is used practically at present.

The present study was designed to observe comparatively the clinical reactions as well as antibody responses in horses injected by various routes with chrome vaccine, and also to find the effect of the vegetable oil adjuvants on immunization with the same vaccine.

Although the experiment was small scale, the conditions were reasonably uniform. Therefore, the results obtained from these experiments seem to be worthy of report.

MATERIALS AND METHODS

1. Horses used in the experiments

All of the examined horses belong to the Kunneppu Branch of the Hokkaido Prefectural Stock Farm. They are 16 foals aged 5~8.5 months and 20 two-year-old

colts; there was an equal number of the two sexes. Most of the horses were of Percheron race, and of Percheron line.

2. Methods of vaccine preparation

Strain of *S. abortus equi*...Strain "Shurei" isolated from gastric contents of aborted fetus. (5/IV '52).

Chrome vaccine...Suspension (1% formol saline) containing 200 mg/ml made from 20 hours agar culture of abortion bacilli was mixed with equal volume of alum chromate solution. After having stood for 5 days at 37°C, this suspension was centrifuged and the sediment was washed two times in normal saline. The final suspension contained 50mg bacilli per ml of saline, added merzonin at the rate of 1:10,000.

Oil chrome vaccine...The chrome vaccine sedimented by centrifugation was mixed by shaking with sterilized peanut oil containing 1% aluminium monostearate. This vaccine contained bacilli of 50 mg/ml

Heat-killed vaccine...Saline suspension containing 100 mg living bacilli per ml was heated at 60°C for 40 minutes and diluted to 50 mg per ml with saline solution containing 0.5% phenol.

Site and dose of injection are indicated in table 1. Intracutaneous or subcutaneous injection was made at the site of left cervical side, and intramuscular injection into *M. glutaeus medius*.

3. Observation on clinical reactions of horses to vaccination

A. Febrile reaction. Temperature was taken two times a day for about one week.

B. Swelling at the site of injection. Long and short diameter, and height of distinct swelling were measured. At the same time, pain and fever as well as suppuration at the site of injection were observed.

C. Leucocytes. Leucocyte count and differential leucocyte count were obtained from only a part of the two-year-old colts.

4. Serological reaction

Sera were obtained from each horse just before and 1/2, 1, 2, 3, 4, and 5 months after vaccination. Methods of serum test are as follows:

O agglutination: Antigen...bacilli treated with alcohol; readings were taken after standing overnight at room temperature following incubation for 2 hours at 37°C.

H agglutination: Antigen...16-18 hours bouillon culture mixed with equal volume of 0.6% formol saline; readings were taken after incubation for 2 hours in water bath at 50°C.

Complement-fixation test: Antigen is supernatant fluid of bacterial suspension centrifuged after boiling; the sensitization was made by incubation at

37°C for 60 minutes, then haemolytic system was added; readings were taken immediately after incubation at 37°C for 30 minutes.

Precipitation: Antigen-carbohydrate fraction of abortion bacilli; the layer test is used; white ring at the junction of antigen and serum was read after incubation at 37°C for 30 minutes.

EXPERIMENTAL RESULTS

1. Clinical reactions

A. Fever

Both maximum temperature and duration of fever are indicated in table 1. In general, in cases injected with heat-killed vaccine, early elevation and rapid fall of temperature are observed. With chrome vaccine injected colts mostly showed the highest fever reaction on the third day after injection, however, the most of the foals showed lower and shorter fever reaction compared with colts.

B. Local reactions at the site of injection

The size of swelling at the site of injection showed significant difference according to the various routes of injection. In cases of subcutaneous injection large swellings occurred. Heat-killed vaccine caused earlier swelling and its rapid disappearance compared with chrome vaccine. In cases of intramuscular injection, local reaction was very slight. Intracutaneous injection caused smaller swelling than subcutaneous. In any site of injection, swelling reactions in the foals were much slighter than those in the two-year-old colts.

Swellings were accompanied by pain and fever at the injected sites especially at the beginning of swelling.

Suppurations occurred in every test group except colts injected with heat-killed vaccine and foals with chrome vaccine (subcutaneous injection). In the cases of intracutaneous injection with chrome vaccine or with oily one, suppurations occurred most frequently even when a minor dose was used. All the pus taken from suppurating sites was examined and it was found that they were abacterial suppurations. These suppurative lesions healed within about one week.

SHIMOJO (1953) has observed that, in human beings or rabbits injected intracutaneously with typhoid vaccine prepared by treatment with alum chromate, an induration or pustule forms at the site of injection. TOBA and his associates (1952) also reported that abacterial

TABLE 1. *Reactions in Horses Injected with Vaccines*

AGE	VACCINE DOSE (mg)	SITE OF INJEC- TION	NO. OF HORSES	FEVER			CHILLS OR SHUD- DERING	LOCAL REACTIONS								
				>39°C	38.5 39°C	Duration of Fever(>38.5°C) Within Over 36 hrs. 48 hrs.		Area of Swell- ing (cm ²) Min.~ Maximum	Mean	Sup- pura- tion	Pain			Fever		
											Severe	Slight	None	Severe	Slight	None
TWO-YEAR-OLD	Chrome 30	s. c.	4	4		1 3	0	162~460	314.5	3	4			3		1
	Chrome 10	i. c.	3	2	1	1 2	0	6.25~42.25	20.25	3	2	1		2	1	
	Chrome 30	i. m.	4	4		1 3	0	0	0	2			4		2	2
	Oil-Chrome 10	i. c.	3	2	1	2(1)	0	121~690	314.3	3	2		1		1	2
	Oil-Chrome 25	i. m.	3	2	1*		2	0~10.5	3.5	1	1		2		2	1
	Heat-Killed 10	s. c.	3	3		3		1	350~500	430	0	1	2			3
5-8.5 MONTHS AFTER BIRTH	Chrome 20	s. c.	4	3	1	3(1)	0	32~180	125	0	3		1	1		3
	Chrome 10	i. c.	3	3		3	0	6.75~12.25	8.58	3			3			3
	Chrome 20	i. m.	3	2	1	3	0	0	0	1			3			3
	Oil-Chrome 10	i. c.	3	1	2	3	0	16~20.25	18.16	3		1	2			3
	Oil-Chrome 20	i. m.	3	3		2 1	0	0	0	1		1	2			3

Figures in each reaction except swelling indicate No. of horses.

Figures in parentheses indicate cases that showed irregular temperature.

* : This case showed less than 38°C.

s. c. : subcutaneous; i. c. : intracutaneous; i. m. : intramuscular.

Area of swelling indicates the maximum in each horse in the course of reaction.

Chrome : Chrome vaccine; Oil-Chrome : Chrome vaccine in oil; Heat-Killed : Heat-killed vaccine.

suppuration occurs in horses injected intramuscularly with their "lipovaccine".

C. Other systemic reactions

Loss of appetite is scarcely observed after injection. Only one colt injected subcutaneously with heat-killed vaccine showed chill and shuddering shortly after injection.

D. Leucocyte count

In a part of the two-year olds leucocytosis was observed for 1~2 days after injection. Cases injected intracutaneously or intramuscularly with chrome vaccine, or subcutaneously with heat-killed vaccine maintained leucocytosis for 3 days at least.

Increase of leucocytes was observed for 2~3 days after injection in the cases not showing swelling or suppuration at the site of injection, but it was not always observed in all the cases of suppuration. (table 2).

These observations are summarized as follows: The mildest local reactions were observed in the cases of intramuscular injection, especially from the point of swelling, pain or fever etc. In the cases subcutaneously injected with heat-killed vaccine, size of swelling was larger than the others but it disappeared rapidly without suppuration. Chrome vaccine and oil chrome vaccine showed high tendency to cause

TABLE 2. *Counts of Leucocytes in Two-year-old Colts after Vaccination*

NAME OF HORSE	VACCINE	DOSE (mg)	SITE	LEUCOCYTE COUNTS				SUPPURATION
				Before Vaccination	Days after Vaccination			
					1	2	3	
Hokuban	Chrome	30	s. c.	16,000	17,300	17,000	13,200	-
Hokuto	"	"	"	10,100	17,700	11,400	8,800	+
Rihoku	"	10	i. c.	12,400	15,700	16,900	16,600	+
Kohoku	"	"	"	9,700	17,600	15,500	10,100	+
Hohoku	"	30	i. m.	12,000	14,800	18,800	19,400	-
Shuhoku	"	"	"	11,000	15,700	17,300	13,900	-
Hokusei	Oil-Chrome	10	i. c.	11,800	16,000	9,200	8,700	+
Hokushun								
Ohoku	"	30	i. m.	18,000	20,900	21,600	22,100	-
Eihoku	"	"	"	11,600	14,200	14,200	15,400	+
Hokkai	Heat-Killed	10	s. c.	11,400	19,200	18,200	17,500	-
Hokutai								
	"	"	"	11,400	16,400	22,400	16,200	-

suppuration when they were injected intracutaneously or subcutaneously. Reactions in foals were generally slighter than those in two-year-old colts.

2. Antibody production

Difference of antibody production owing to difference among the kinds of vaccine, the sites of injection, or other factors was examined mainly by means of O agglutinin titres. At the same time, rise and fall of H agglutinins, complement-fixing antibodies and precipitins were observed and compared.

A. O agglutinins

Table 3 indicates the geometric mean of agglutinin titres of each test group and figure 1 illustrates graphically the proportion of rise and fall of agglutinin titres in table 3.

According to this table, it is obvious that on an average O agglutinins develop into maximum titres a half month after injection, then they decrease rapidly. O agglutinin titres after 4~5 months are slightly higher, or lower than those before injection despite the difference of vaccines or the sites of injection.

It is an interesting fact that, in colts two-year-old, O agglutinin titres in the 5th month after injection became rather lower than those before injection. In any site of injection, O agglutinin titres of chrome

TABLE 3. O Agglutinin Titres in each Test Group

AGE	VACCINES	DOSE (mg)	SITE	NO. OF HORSE	O AGGLUTININ TITRES (GEOMETRIC MEAN)						
					Before Inject.	Months after Injection					
					1/2	1	2	3	4	5	
TWO-YEAR-OLD	Chrome	30	s. c.	4	200	1346	800	476	283	336	50
	"	10	i. c.	3	252	2025	800	1008	504	504	119
	"	30	i.m.	4	336	3810	1600	1353	1131	476	159.3
	Oil-Chrome	10	i. c.	3	200	1270	400	400	504	159.3	39.7
	"	25	i.m.	3	317.4	2016	800	800	635	252	79.4
	Heat-Killed	10	s. c.	3	252	1131	635	504	400	363	63
5-5.5 MONTHS AFTER BIRTH	Chrome	20	s. c.	4	141.4	5381	800	476	241	168	
	"	10	i. c.	3	158.8	2540	504	400	400	126	
	"	20	i.m.	3	126	1600	200	504	252	100	
	Oil-Chrome	10	i. c.	3	200	468	504	200	159.3	200	
	"	20	i.m.	3	252	1270	400	504	317.4	252	

vaccine emulsified in peanut oil seem to be generally lower than those of chrome vaccine. Without distinction of age, the cases intracutaneously injected with chrome vaccines showed, on an average, the moderate rise of O agglutinin titres. Two-year-old colts injected intramuscularly sustained relatively high O agglutinin titres both in chrome vaccine and in the same vaccine in oil.

According to figure 1, the proportion of descent of O agglutinin titres from a half to one month after injection is more remarkably large in foals injected with chrome vaccine than in the two-year-old colts. Excepting this point, rise and fall of O agglutinins showed the same tendency in both groups of foals and colts.

B. H agglutinins

As rise and fall of antibodies seems to be uniform in every test group irrespective of kind of vaccine or of the site of injection, groups injected intracutaneously with each kind of vaccine are taken as the source of the data in table 4. By this table, antibody titres in foals may be efficiently compared with titres in two-year-old colts under the same conditions.

In table 4, H agglutinins in every case were negative at 1:50 before injection, but they developed with O agglutinins after injection. A small quantity of H agglutinins in two-year-old colts was still present in 5th month after injection, while H agglutinins in foals seemed to fall below 1:25 in 3rd month after vaccination. From these observations, it may be stated that H agglutinins in foals disappear earlier than those in the two-year-olds.

C. Complement-fixing antibodies

Titres of complement-fixing antibodies rise to maximum with O or H agglutinins. Two-year-old colts still sustained the maximum titres of complement-fixing antibodies or titres near to maximum in the 4th month after injection. However, rise and fall of complement-fixing titres in foals generally go parallel with those of H agglutinin titres, and these antibodies disappear absolutely in 3rd month after injection.

D. Precipitins

In ascending stadium, precipitins are produced in parallel with other antibodies. However, precipitin production was not observed at all in 4 of 12 cases. Precipitins seem to disappear rapidly compared with other antibodies. In only one foal, precipitins were found to be still present 2 months after injection.

Figure 1. Rise and Fall of O Agglutinin Titres (Geometric Mean).

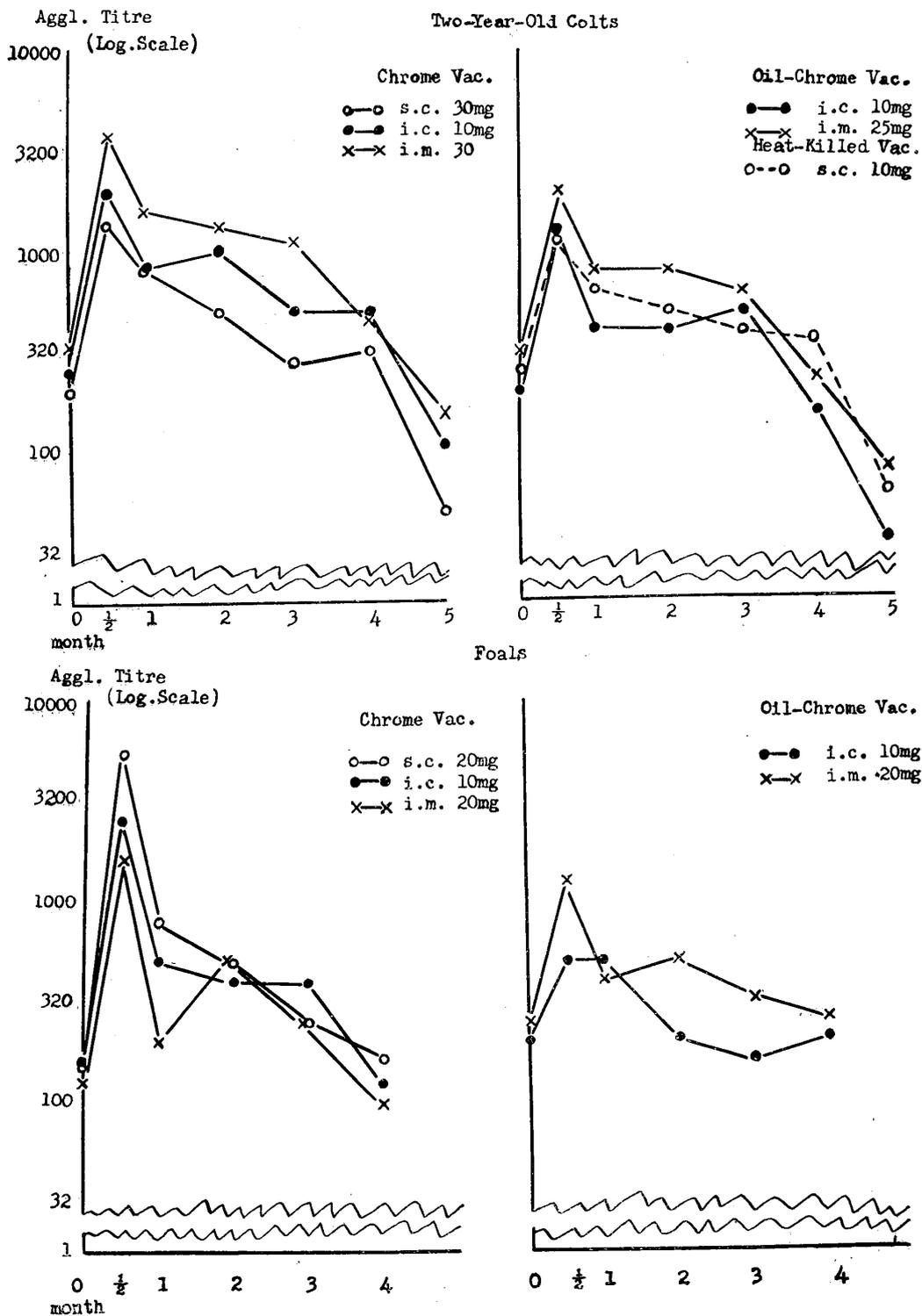


TABLE 4. *Antibody Titres in Foals and Colts*

AGE	VACCINES DOSE(mg) SITE	NAME OF HORSE	O AGGLUTININ TITRES							H AGGLUTININ TITRES						
			Before Inject.	Months after Injection					Before Inject.	Months after Injection						
				1/2	1	2	3	4		5	1/2	1	2	3	4	5
TWO-YEAR-OLD	Chrome	Rihoku	200	3200	1600	1600	400	800	200	1:50	800	800	400	100	200	50
		10	200	800	400	800	400	200	100	—	400	400	200	200	100	100
		i. c.	400	3200	800	800	800	800	200	—	1600	800	200	100	50	50
	Oil-Chrome	Hokusei	200	800	400	400	800	50	25	—	800	400	100	50	50	50
		10	200	1600	400	400	200	200	50	—	1600	200	100	200	100	50
		i. c.	200	1600	400	400	800	400	50	—	400	100	100	50	25	25
8 Months	Chrome	Hidehagi	200	3200	400	800	800	100	—	800	200	25	—	—	—	
		10	200	3200	400	400	200	100	—	400	—	25	—	—		
		i. c.	100	1600	800	200	400	200	—	800	—	—	—	—		
7.5 Months	Oil-Chrome	Eitoh	200	6400	800	400	200	200	—	200	100	25	—	—		
		10	200	400	400	100	100	25	—	200	400	25	—	—		
		i. c.	200	400	400	200	200	400	—	100	200	25	—	—		

AGE	VACCINES DOSE(mg) SITE	NAME OF HORSE	COMPLEMENT-FIXING ANTIBODIES							PRECIPITIN				
			1:2						1:5					
				16	8	4	2	1		80	40	20	10	5
TWO-YEAR-OLD	Chrome	Rihoku	—	16	16	8	8	16	—	80	80	80	20	—
		10	—	8	4	8	4	2	—	40	5	—	—	
		i. c.	—	16	8	4	2	4	—	40	40	—	—	
	Oil-Chrome	Hokusei	—	8	16	8	4	8	—	—	—	—	—	
		10	—	4	8	4	2	2	—	—	—	—	—	
		i. c.	—	8	8	8	2	8	—	40	—	—	—	
8 Months	Chrome	Hodehagi	—	2	—	—	—	—	—	20	—	—	—	
		10	—	2	4	2	—	—	—	5	—	—	—	
		i. c.	—	/	/	2	—	—	—	—	10	—	—	
7.5 Months	Oil-Chrome	Eitoh	—	8	8	2	—	—	—	160	20	—	—	
		10	—	4	2	—	—	—	—	—	—	—		
		i. c.	—	2	2	2	—	—	—	—	—	—		

Titres of Complement-Fixing Antibodies : Positive complement fixation at each dilution of sera.
 Titres of Precipitin : Positive precipitate at each dilution of antigen.

DISCUSSION AND CONCLUSIONS

In a previous paper the present authors suggested that the use of chrome vaccine in horses may be recommendable from the viewpoint of the small toxicity and the good antigenicity against mice.

When this chrome vaccine was tested in two-year-old colts, the group subjected to intramuscular injection showed minor local reactions and increased immune response. Accordingly, it is obvious that intramuscular injection is suitable for chrome vaccine as well as for commercial "lipovaccine" which is practically injected by this route. However, abacterial suppuration at the sites of injection occurs frequently in horses injected with chrome vaccine. Therefore, for the purpose of practical use of this vaccine, improvements related to this point seem to be desirable.

Many studies have been carried out on the question whether intracutaneous injection of typhoid vaccine in human beings is effective or not.

SILER and DUNHAM (1939) recommend a single dose of the phenolized vaccine injected intradermally; they say this provides an adequate stimulus, and gives rise to less local and systemic reaction than the larger dose which is usually given subcutaneously. In the experiments described in the present paper, no special favourable effect of intracutaneous injection was observed either in the clinical reactions or in antibody productions. However, further studies on intracutaneous injection with a minute dose are needed for the observation of clinical reaction and antibody production.

ANDO (1951) suggested that the reduction of toxic reaction of his typhoid chrome vaccine is induced by the slow dissolution of toxic substances from bacilli which are leathered by the treatment with alum chromate. This idea corresponds to our results that the heat-killed vaccine injected subcutaneously causes rapid fever and local reaction, and quick disappearance of these reactions compared with the delay of reaction in chrome vaccine. The delay of systemic and local reaction in chrome vaccine may depend upon the difficulty of absorption of leathered bacterial substance. TOBA and his associates (1952) prepared "lipovaccine" of abortion bacilli in order to inhibit the rapid absorption of vaccine so as to make the stimulative effect of an antigen continue for a longer time.

Regarding the severity of toxicity of both heat-killed and chrome

vaccine, as mentioned above, the former causes rapid and the latter delayed reaction. The chrome vaccine tends to cause suppuration at the site of injection. So that it is not easy to distinguish which vaccine is more beneficial than the other. On the other hand, the rise and fall of O agglutinin in chrome vaccine subcutaneously injected, showed almost the same result as in heat-killed vaccine. Moreover, chrome vaccine emulsified in peanut oil showed neither longer duration of antibody production nor higher titres of O agglutinin than chrome vaccine only. It rather showed lower titres than chrome vaccine. It is impossible to explain whether this effect depends on the defect of vaccine preparation (lanoline-like substance was not used) or on the scant effect of peanut oil adjuvants. FREUND et al. (1948) noted a slight effect of peanut oil on typhoid bacilli in contrast to paraffine oil.

O, H agglutinins and complement-fixing antibodies appear after injection without exception and maintained their high titres for a considerably long time, while precipitin production is much restricted compared with the above antibodies. In some cases precipitins are not produced and they disappear rapidly even in positive precipitin cases.

HAMADA (1953) has reported that foals less than 60~70 days after birth were lacking in capacity for agglutinin or precipitin production, while more than 148 days old foals vaccinated with heat-killed abortion bacilli showed as complete capacity for antibody production as did the adult horses. The present authors from long observations on rise and fall of antibodies found a significant difference of antibody production between 5~8.5 months old and two-year-old colts. It was found that in two-year-old colts, antibody titres in the 5th month after injection did not always descend to the counts obtained before injection, while in foals they fell very rapidly; H agglutinins and complement-fixing antibodies disappear completely in the 3rd month after injection of killed abortion bacilli.

In order properly to test the potency of vaccine, it is most desirable to test directly whether individuals immunized are able to be protected against infection or not. Secondly, mouse protection test by sera obtained from immunized individuals may be practical. It is well known that this test is generally used in typhoid vaccine. In the present experiments, only the production of antibodies other than antibacterial antibodies was observed, the authors believe that potency test of killed vaccine of abortion bacilli should be performed also by means of the above methods *in vivo* in future.

SUMMARY

1. Intramuscular injection of chrome vaccine in horses was favourable from the viewpoint of clinical reactions and antibody production.
2. Heat-killed vaccine injected subcutaneously caused early local and systemic reaction and its rapid disappearance compared with chrome vaccine.
3. Chrome vaccine and oil chrome vaccine showed high tendency to cause suppuration when they were injected intra- or subcutaneously.
4. Foals generally react more slightly than two-year-old colts.
5. Marked increase of antigenicity was not observed in chrome vaccine.
6. Peanut oil adjuvants without lanoline had no enhancing effect on chrome vaccine.
7. Duration of antibody production in 5~8.5 months old foals is shorter than that in two-year-old colts.

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