Title	THE BEHAVIOR OF THE RABBIT AGAINST INFECTION WITH SALMONELLA ABORTIVOEQUINA
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Citation	Japanese Journal of Veterinary Research, 8(1-4), 237-250
Issue Date	1960
DOI	10.14943/jjvr.8.1-4.237
Doc URL	http://hdl.handle.net/2115/4682
Type	bulletin (article)
File Information	KJ00002373274.pdf

Instructions for use

THE BEHAVIOR OF THE RABBIT AGAINST INFECTION WITH SALMONELLA ABORTIVOEQUINA

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(Received for publication, June 6, 1960)

INTRODUCTION

The behavior of the horse against infection with *Sallmonella abortivoequina* was previously investigated in detail by KASAI, HIRATO and co-workers[4,6,7], however, there seem to be many problems concerning diagnosis, vaccination, etc. remaining yet to be studied.

It goes without saying that from the standpoint of host-parasite relationship, the horse may be the most suitable animal for these experiments, but it is nearly impossible to employ them because of their high value. Accordingly, the choice of some other small experimental animals which will serve in substitution for the horse have been eagerly investigated by many workers.

Up to this time, for the experimental studies of this disease, mice have been used in general by many workers in this country: OCHI, SOEKAWA, HIRATO and co-workers[8,9], TOBA and so on.

However, the susceptibility of the mouse to infection with *S. abortivoequina* seems to be pretty high to every route of inoculation compared with that of the horse. For this reason, SATO et al. in this laboratory, several years ago made preliminary observations on rabbits; they noted that the rabbit showed a low susceptibility somewhat resembling that of the horse to this organism without causing septicemia which is frequently seen in the mouse.

Accordingly, the author made more systematic investigations on the behavior of the rabbit against infection with this organism.

The present report describes the results of observations on rabbits inoculated with *S. abortivoequina* from bacteriological, clinical as well as pathological points of view.

MATERIALS AND METHODS

1. Experimental animals: Thirty-two apparently healthy albino rabbits, each weighing approximately 1.5 kg, were used.

2. Strains used : The strains of *S. abortivoequina* used for the inoculation were freshly isolated ones from gastric contents of aborted equine fetus. For the serological tests strain "Urahoro" (referred to as "E 9" hereafter), stocked in this laboratory, was employed as the antigen.

3. Route of inoculation : S-form colony, isolated from gastric contents as noted above, was cultivated at 37°C for 18 hours in broth. About 2~3 ml of culture dilutions each of which contained the desired number of organisms were perorally inoculated by the use of glass pipettes. Concerning the inoculated doses, description will be offered in detail in the following chapter.

4. Serological reactions :

O-agglutination : Somatic antigen was "E 9" bacilli treated with alcohol ; readings were made after material had been left standing overnight at room temperature following incubation at 37°C for 2 hours.

H-agglutination : "E 9" strain was used as the flagellar antigen. It was prepared from 8~9-hour broth cultures mixed with equal volume of 0.6% formol saline ; readings were made immediately after incubation in water bath at 50°C for 2 hours.

Precipitation : The carbohydrate fraction of "E 9" bacilli obtained by the method of SASAKI was used as the antigen ; test was performed by overlay method at 37°C for 10~20 minutes.

Complement fixation : The antigen was the clear supernatant obtained by centrifugation of the boiled bacterial suspension. The mixture of antigen, test sera and complement was placed in water bath at 37°C for 60 minutes, thereafter, the hemolytic system was added and placed in water bath at 37°C for 30 minutes, readings were made immediately after this. In this test, all sera were inactivated in a water bath at 62°C for 30 minutes. Doses or units of each component are similar to those used in the procedure of the American Army Medical School.

Hemagglutination : The test was carried out chiefly following the method described by VERNON et al. Antigen was prepared by sensitizing the tannic acid treated sheep red cells with above mentioned polysaccharide antigen ; readings were made by pattern on the bottom of tube after material had stood 3 hours at room temperature.

5. Bacteriological observations : Cultivations of the organism from the circulating blood were made once every day or every other day during 10 days after inoculation, and thereafter once every week. In some cases, cultures of feces were made every day for 10~15 days after inoculation.

6. Clinical and hematological observations : The body temperature was measured in rectum twice daily and body weight was examined once every other day for about the first 10 days and thereafter, at 4~7 days interval. General health conditions were also carefully observed.

Hematological observations were made once before and once every other day for 1 week after inoculation and thereafter at least once a week.

At the end of the observations, the dissected animals were macroscopically examined and various organ materials as shown in table 6 were cultivated directly or by enrichment method in order to allow investigation of the distribution of the organism in animal bodies.

EXPERIMENTAL RESULTS

A) General descriptions of the susceptibility of the rabbit to the organism

Total 32 rabbits were divided into 12 groups, each comprised of 1 to 6 individuals.

As previously mentioned, the inoculation of the organism into the rabbit was carried out exclusively peroral. The number of organisms which were inoculated to rabbits of each group will be seen from table 1. An outline of clinical and bacteriological responses of rabbits to this organism following inoculation is also indicated in this table.

TABLE 1. *Inoculation of S. abortivoequina into Rabbits and
Their Clinical and Bacteriological Responses*

GROUP OF RABBITS	DOSE ×10⁸	ROUTE	FEVER REACTION	OTHER CLINICAL SIGNS	BACTE- REMIA	DEATH	ANTIBODY RESPONSES	DETECTION OF INOCULATED ORGANISM
1	0.5	Per Os	2/2*	2/2	0/2	0/2	2/2	0/2
2	1.6	,,	3/5	4/5	0/5	0/5	5/5	1/5
3	6.4	,,	4/5	5/5	1/5	0/5	5/5	3/5
4	10	,,	2/2	2/2	0/2	0/2	2/2	1/2
5	15	,,	3/6	5/6	3/6	1/6	5/6	5/6
6	20	,,	2/2	2/2	1/2	1/2	2/2	1/2
7	40	,,	2/2	2/2	1/2	0/2	2/2	2/2
8	60	,,	1/2	2/2	0/2	1/2	2/2	2/2
9	100	,,	2/2	2/2	0/2	0/2	2/2	0/2
10	170	,,	0/1	1/1	0/1	0/1	1/1	0/1
11	200	,,	2/2	2/2	0/2	0/2	2/2	1/2
12	250	,,	1/1	1/1	1/1	0/1	1/1	0/1

Note : * No. of positive/ No. of examined

Generally speaking, it will be seen in the table that the clinical manifestations of rabbits against infection with this organism seems to be not very noticeable.

Only one rabbit which had received 2,000 million bacilli died from primary septicemia, while the other animals which had been inoculated with more than 10 times larger dose than the above one, resisted without showing bacteremia. However it is clear that almost all rabbits which did not show any symptoms were infected by this organism slightly, because of their positive immunological responses. From these results, the author concluded that rabbits have considerably high susceptibility to this organism; however the behavior of the rabbit against infection with *S. abortivoequina* was different from that of the mouse.

B) Clinical and hematological observations

1) Febrile reaction : Fever attack over 40°C was observed in 23 out of the 32 cases (71.9%). The other cases also showed the slight fever reactions of 39.5°C or more; these temperatures seem to be somewhat abnormal ones. Accordingly it is clear that all rabbits employed showed febrile responses.

The type of febrile reactions manifested by the rabbit could roughly be divided into the following 2 groups : (1) The group which showed febris ephemera after a certain incubation period (1~9 days) and then turned to irregular fever (10/23=43.5%) or once more febris ephemera in 2~10 days (5/23=21.7%) after an alleviation of the fever. (2) The group which showed febris continua with a duration of 2~10 days and then turned to irregular (5/23=21.7%) or to febris ephemera (3/23=13.1%) after an alleviation of the fever.

2) The incubation period : Assuming that the incubation period means the time from inoculation to fever attack, it is clear that these periods vary extremely from each other, as will be seen in table 2.

TABLE 2.   *Incubation Period judging from Febrile Reactions and Their Relation to Doses Inoculated*

INOCULATED DOSES	NO. SHOWED FEBRILE REACTION/ NO. TESTED	INCUBATION PERIOD IN DAYS									AVERAGE INCUBATION PERIOD	RATE OF POSITIVE FEBRILE REACTION
		1	2	3	4	5	6	7	8	9		
×10⁸ 0.5–6.4	8/12					2	3	2	1		days 6.3	% 66.6
10–60	10/14		1	1	3		2		2	1	5.4 } 5.3	71.4 } 71.9
100–250	5/ 6	2			1	1	1				3.4	83.3

More than half of the rabbits (13/23) showed incubation periods ranging from 4 to 6 days, although they varied from 1 to 9 days after inoculation (5.3 days on average).

These differences seem somewhat to be influenced by the inoculated doses, as will be seen in the table.

3) Hematological observations : After inoculation, wide fluctuations of the leucocyte count were generally observed. The course of these fluctuations was divided into following 3 types : (1) The group which showed some increase during 2~3 days after inoculation, abruptly turned to leucopenia with the first attack of fever, then turned to leucocytosis soon after the alleviation of fever, and thereafter become normal (11 cases, 50%). (2) In this group, decrease in counts occurred soon after inoculation ; during the period of 1~2 weeks, leucopenia was observed to occur abruptly, and after the alleviation of fever the cases gradually turned to normal (6 cases, 27.3%). (3) The group which did not show the above-described patterns, although the fluctuations of the leucocyte count were obviously severe making a "saw-toothed" graph pattern (4 cases, 18.2%).

The nucleus-shift of neutrophile leucocyte : The nucleus-shift-to-the-left was observed clearly in almost all cases. From the next day after inoculation, the nucleus-shift began to move gradually and reached the maximum displacement at the alleviation of fever.

Usually, such cases tended to need long time for recovery, and their periods seemed to run in parallel with those of declining of various antibody titers.

4) Other clinical signs : Diarrhoea was not very severe among the rabbits but the loss of appetite was marked in almost all cases. Reduction of body weight was also observed in 28 out of the 32 cases (87.5%).

C) Bacteriological observations before death

1) Bacteremia: In the febrile stage, the organisms were detected directly from their circulating blood in only 7 out of the 23 rabbits (30.4%) which showed febrile reactions of over 40°C. However, there may exist more intimate relationships between bacteremia and fever attack, which may possibly be demonstrated by the improvement of the cultivation method, and by finding out the best time for detection.

Bacteremia usually began with development of the fever attack, continued during the period of high fever and disappeared with alleviation of the fever. In the present cases, the length of time of the appearence of bacteremia was as follows: 1 day 4 cases, 2 days 2 cases, and 4 days 1 case.

2) Excretion of organisms in feces: Observations were carried out on 10 cases of the rabbit for the period of 10~15 days after inoculation. Despite the cultivations by enrichment method, detection of organism was in just one case (No. 121).

D) Antibody responses

1) Outline of rise and fall of the various antibodies: The production of antibodies was observed in almost all cases, their behaviors were not similar to each other. In the majority of cases, O (somatic)- and H (flagellar)-agglutinin developed rapidly and reached the maximum at the 3rd~4th weeks after inoculation, while CF (complement fixing) antibody tended to rise slowly. Precipitin took a middle course. Concerning the appearances of antibodies, there seem to be no differences corresponding to doses inoculated. Figures 1 and 2 indicate the production of each antibody after inoculation.

FIGURE 1. *Appearance of Each Antibody after Inoculation*
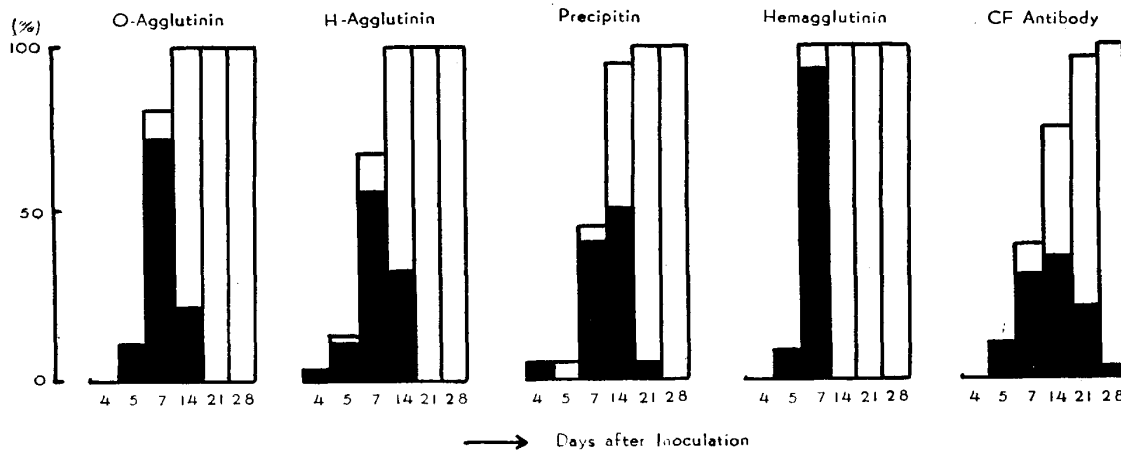
Notes: ■ ··· Indicates the percentage of the newly formed antibody
in each day of inspection
▢ ··· Indicates the total percentage of antibody formation
which was detected till the day of inspection

During the period of the 4~8th weeks after inoculation which was assumed for the middle stage of infection, almost all antibody titers reached their maximum levels.

It can scarecely be said that the individual antibody titers and their persistence were

FIGURE 2. *Rise and Fall of Each Antibody Produced in Rabbits*

Days   after   Inoculation
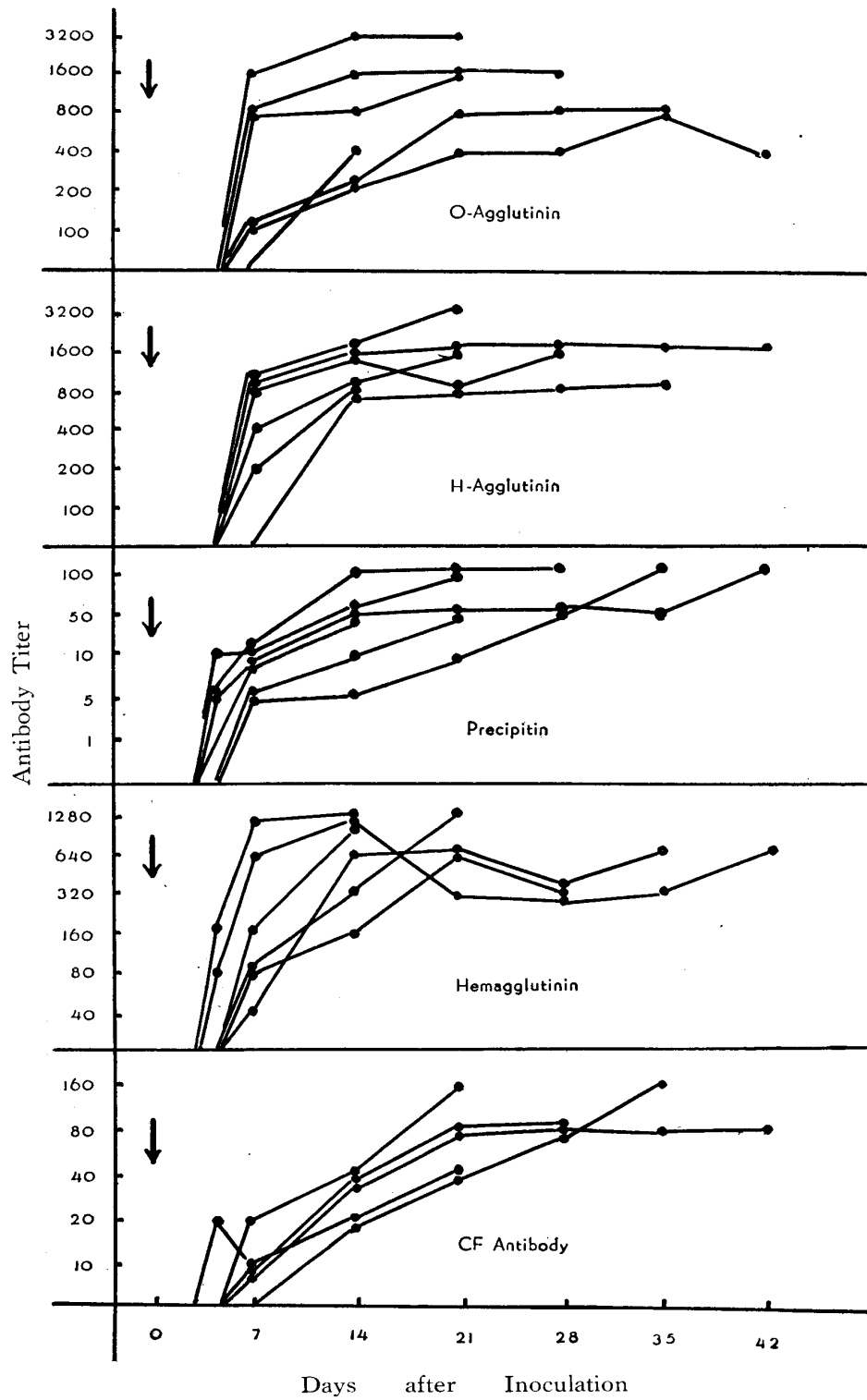
Note : The above data were obtained from the test groups of 6, 7 and 8

largely influenced by the activities of the invaded organisms in animal bodies. All kinds of antibodies generally maintained their equilibrium titers or fell very slowly.

At the 8th week or later after inoculation which was assumed to be the convalescent stage, titers of all kinds of antibodies generally began to fall, however, there were some differences in the characters of their fall. Hemagglutinin, precipitin and CF antibody fell somewhat earlier than the others; the former two antibodies disappeared completely, while CF antibody persisted at very low titer.

Fall of O- and H-agglutinin are very slow; the author could not observe even one case which showed complete disappearance within the observation period (18 weeks).

In the course of experiments, it became apparent that there were some cases which maintained relatively high precipitin titer for a long period, despite the gradual decrease of the other antibodies. In these cases, post-mortem examination revealed the presence of the organisms. These findings seem to be very important for serological diagnosis as has already

FIGURE 3.   *Relation between Antibody-Titer and Inoculated Dose at the 5th Week after Inoculation*

Inoculated Doses ($\times 10^8$)
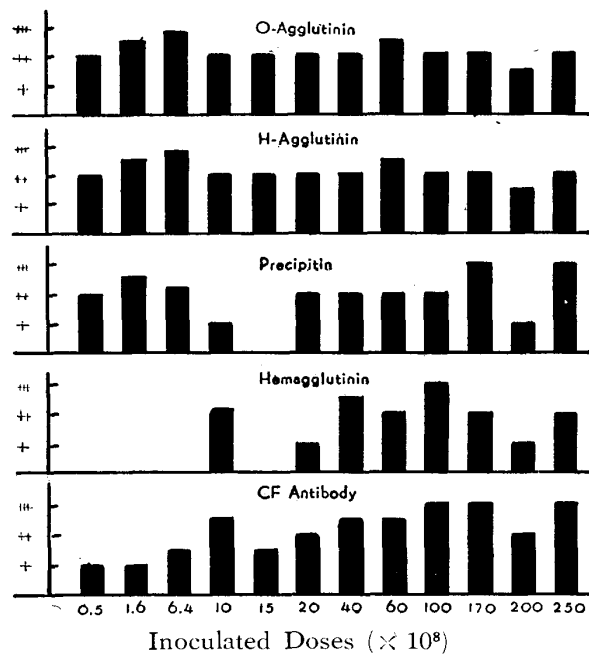
Notes :   1.   Hemagglutination test was not made on the cases which had been inoculated with small doses

2.   +, ++, +++ mean the following antibody titers :

	H & O	Pr	Hem	CF
+	1 : 100~1 : 400	1 : 1	1 : 80~1 : 320	1 : 10~1 : 20
++	1 : 800~1 : 1600	1 : 5~1: 10	1 : 640	1 : 40~1 : 80
+++	1 : 3200	1 : 50	1 : 1280	1 : 160

Notes :   H···H–Agglutinin   O···O–Agglutinin   Pr···Precipitin
Hem···Hemagglutinin   CF···CF Antibody

been emphasized by HIRATO et al.[10].

2) Relation between antibody titer and inoculated dose: As previously stated, it is certain that in each case the appearence of the antibody is not affected by the doses inoculated. Then, the antibody titers were examined at the 5th week after inoculation to ascertain whether or not there were any differences according to the inoculated doses.

Each average titer was calculated and illustrated in figure 3. However, the author also could not find out any particular correspondence between the antibody titers and doses inoculated as will be seen from figure 3.

3) The antibody titer and result of cultivation: By using the 12 cases which were slaughtered at and after 6 weeks following inoculation, attempt was made to discover whether there are any correlations between antibody titers and preservation of organisms in animal body or not. The results are shown in table 3.

TABLE 3. *Relationship between Results of Cultivation and Antibody-Titer*

RABBIT NO.	INOCULATED DOSES	DAYS AFTER INOCULATION	DETECTION OF ORGANISMS	ANTIBODY-TITER				
				Pr	Hem	O	H	CF
	$\times 10^8$	Weeks						
23	1.6	6	—	+	×	++	+	+
24	,,	8	+	++	×	++	+	+
25	,,	10	—	—	×	+	+	—
33	6.4	6	+	+++	×	+++	++	+
34	,,	8	—	—	×	+++	++	+
35	,,	10	—	—	×	++	+	+
51	15	10	+	×	×	+	++	+
53	,,	8	+	×	×	+	+	+
54	,,	6	+	×	×	++	++	++
62	20	6	—	+	+++	+	++	++
101	170	18	—	—	—	+	+	+
121	250	18	—	+	—	+	+	+

Notes: Abbreviations for antibody titers such as —, +, ++, +++ are the same as in fig. 3.   ×···Not examined

According to this, it is sure that the high precipitin titer indicates the presence of the organisms in animal bodies although the cases examined were not very many. However, the high titers of the other antibodies do not always indicate a reservoir of the organisms.

E) Post-mortem findings

1) Macroscopical changes: Enlargement of the mesenteric lymphnode was observed markedly in 17 out of the 32 cases (53.1%) while in the other organs, the grade of enlargement was found to be high in the following descending order: the spleen, liver, kidney and cervical lymphnodes.

2) Distribution of the organisms in the rabbit body: Regarding the 16 cases which showed positive cultures, the distribution of the organisms was examined. The results are

TABLE 4. *Distribution of S. abortivoequina in the Tissues and Organs of Infected Rabbits*

RABBIT NO.	INOCULATED DOSE ($\times 10^8$)	DAYS ELAPSED FROM INOCULATION TO SLAUGHTER (Weeks)	Liver	Kidney	Slpeen	Heart	Lung	Heart Blood	Peritoneal Fluid	Bile	Urine	Testicle	Ovarium	Uterus	Bladder	Thyroid Gland	Thymus Gland	Submaxillary Lymphnode	Pharyngeal Lymphnode	Axillary Lymphnode	Bronchial Lymphnode	Inguinal Lymphnode	Iliac Lymphnode	Mesenteric Lymphnode	Stomach	Duodenum	Jejunum	Ileum	Caecum	Colon	Rectum
24	1.6	8	—	—	+	—	—	—	—	—	—	—	•	•	—	—	—	+	—	—	—	—	—	+	—	—	—	—	—	—	—
31	6.4	2	+	—	‡	—	‡	—	•	•	—	•	—	—	•	—	—	—	—	—	•	—	—	+	—	—	—	—	—	—	—
32	″	4	‡	—	‡	—	—	—	‡	—	—	•	—	—	—	—	—	‡	—	—	—	—	—	‡	—	—	—	—	—	—	—
33	″	6	—	—	+	—	—	—	—	—	•	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	‡	‡	‡	—
42	10	5	—	—	—	—	—	—	—	—	—	—	•	•	—	—	—	‡	—	—	—	—	—	+	—	+	‡	+	‡	‡	+
51	15	10	—	—	+	—	—	—	—	—	•	—	—	—	+	—	—	+	—	—	+	—	—	+	—	—	—	+	‡	—	—
53	″	8	—	—	+	—	—	⊕	—	—	—	—	—	—	—	—	—	+	—	—	—	—	—	‡	—	—	—	—	‡	‡	‡
54	″	6	+	—	+	—	—	⊕	—	—	—	—	•	•	—	—	•	—	—	—	—	—	—	+	—	—	—	—	—	—	—
55	″	4	—	—	—	—	—	⊕	—	—	•	—	—	—	—	—	—	—	—	—	—	—	—	‡	—	—	—	—	—	—	—
56	″	2	—	—	+	—	—	⊕	—	+	—	—	•	•	—	—	•	+	—	—	+	—	+	+	—	—	—	—	—	—	—
61	20	2	‡	+	‡	—	‡	—	—	+	—	•	•	•	•	•	—	‡	—	—	+	—	+	‡	+	—	—	—	—	—	—
71	40	3	+	—	+	—	—	—	—	—	—	•	•	•	—	•	•	‡	—	—	—	—	—	‡	—	—	—	—	—	—	—
72	″	5	—	—	+	—	—	⊕	+	—	—	•	+	—	•	—	—	—	—	—	•	—	—	‡	—	—	—	—	—	—	—
81	60	4	—	—	‡	—	—	—	‡	•	—	•	+	—	‡	+	—	+	‡	—	—	—	—	‡	—	‡	—	‡	—	—	—
82	″	3	—	—	+	—	—	—	•	•	•	•	•	•	•	+	‡	‡	—	+	+	—	—	‡	·	—	—	+	+	—	—
111	200	5	—	—	—	—	—	—	•	•	—	—	•	•	—	—	—	—	—	—	—	—	•	‡	—	—	—	—	‡	—	—

Notes: + ... Positive result in direct cultivation
+ ... Positive result after enrichment
⊕ ...Positive result in the circulating blood
— ... Negative result
• ...Not examined

FIGURE 4.   *General Responses of Rabbit No. 121*
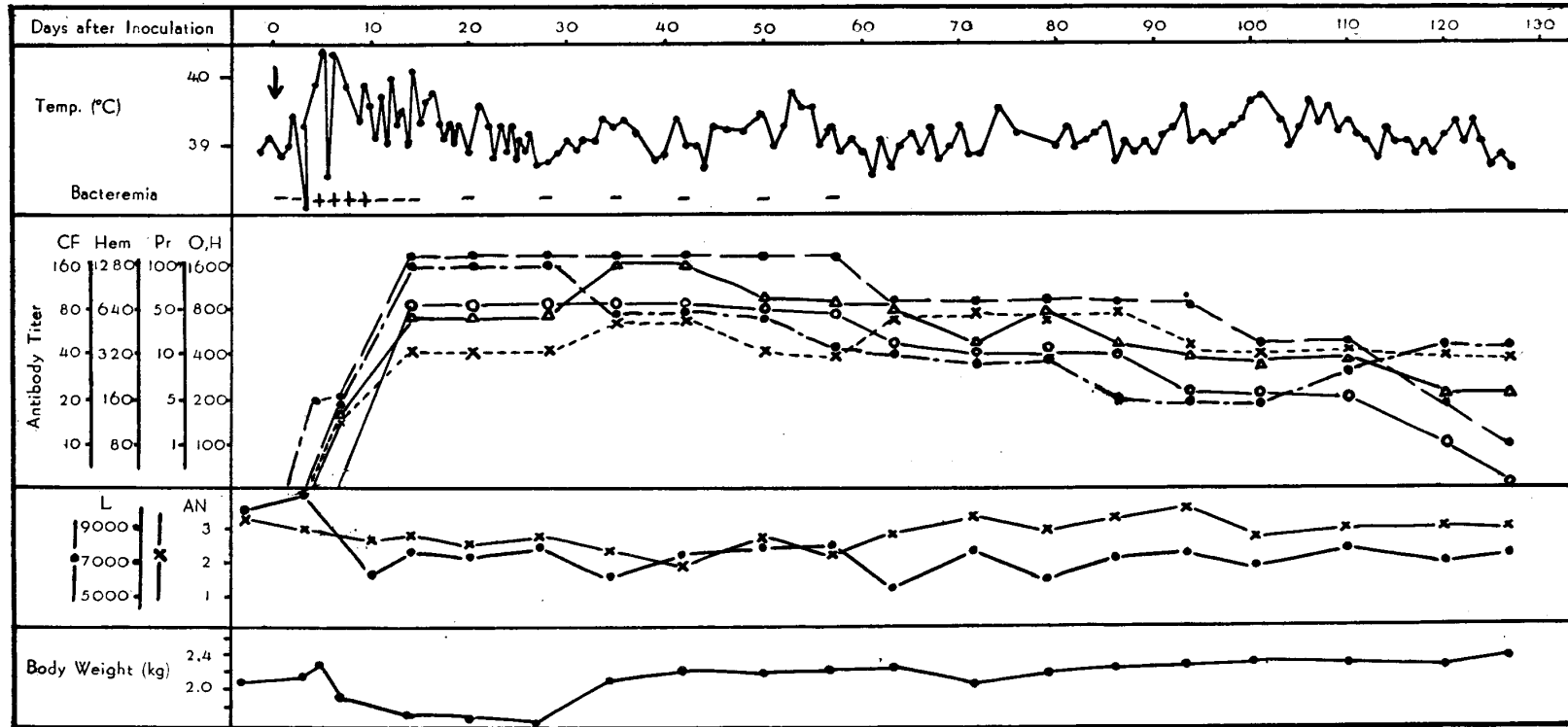
Notes : ------ X ------- ··· O   ———•——·—— ···H   —— —·— ··· Pr   ——△—— ··· CF   ——— ○——— ··· Hem

L ··· Leucocyte Count   AN ··· Average Nuclear Count of Leucocytes

shown in table 4. From the table, it will be noticed that mesenteric lymphnode harbored the organisms in almost 100 percent of the cases. This fact may be due to the peroral route of inoculation which was always employed in this experiment.

Among the 7 cases which showed primary bacteremia, the organisms were detected in 5 cases (Nos. 54, 56, 61 & 72) in several organs and in the other 2 cases they were not detected at all similar to those which did not show bacteremia throughout the observation period. It is especially interesting that, one case (No. 121) of the latter had completely cleared up the organism from her body, despite showing bacteremia during 4 days.

Although this was only one case, it seems to indicate the typical course of the disease — infection and convalescence — during the experimental period.

Accordingly the primary bacteremia may not exert influence on the distribution of organisms in animal body as far as examined at the 2nd~6th weeks after the end of bacteremia.

DISCUSSION

From the experiments on the pathogenicity of this organism for the horse, NAMIKAWA et al. and FUKANO confirmed that the adult horse showed only slight susceptibility, while HIRATO and co-workers[4,6,7] and HAMADA[3] stated that abortion bacilli, even in small dose, could cause abortion in pregnant mares, and moreover HIRATO & HAMADA, HAMADA[3] and KUTII et al. reported that this organism could cause somewhat serious infection in foals.

From the present data, the behavior of rabbits against infection with this organism seemed to be similar to that of foals though it was in somewhat low degree.

As to the rise and fall of the antibodies, especially in O-agglutinin and CF antibody, it was quite similar to that of the horse. However, in the cases of rabbits the author observed the high development of H-agglutinin, contrary to the data on the horse offered by many authors who reported no or a very low development of this antibody. With respect to this finding, SATO et al. already recognized the low development of H-agglutinin in rabbits, however, further examinations may be necessary for emphasizing of this finding.

As regards the precipitation test, HIRATO et al.[10] suggested that rise and fall of precipitin reacting with bacterial carbohydrate antigen was considerably in parallel with the persistence of the organism in animal body. In the present report, some rabbits which manifested strong positive precipitation test, harbored the organisms.

Although the rise and fall of hemagglutinin generally ran in parallel with rise and fall of the other antibodies, it was of especial interest that this antibody was detected in somewhat earlier stage of the disease and showed the tendency to run in parallel with occurrence of precipitin at the convalescent. Therefore, hemagglutination test may be useful for rapid detection of this disease.

On the other hand, the results of examination of the bacterial distribution in the body of rabbits also showed a tendency quite similar to that in horses which has been reported by HIRATO et al.[11], HAMADA[2,3] and KUTII et al.

In the present cases, a fever attack was observed in high percentage, though the majority of attacks showed febris ephemera.

As regards the incubation period, the present data on rabbits almost completely coincided with the report by HAMADA[3] which described that all foals which were inoculated with a large dose of the organism revealed a rise of temperature within 2 days, however, foals with a small dose, took 6 to 12 days.

Moreover, hematologically marked decrease of nuclear segmentation of leucocytes, namely, shift-to-the-left was especially noticed in almost all cases. It is not meant to say that this finding is one of the characteristic signs of the horse affected with this organism.

The author has made no histopathological observations yet to conifirm catarrhal enteritis with swollen Peyer's patches which were observed on experimental horses by KUTII et al. and HAMADA[3]. However, from the data offered by these authors who stated that such histopatholoigcal change indicates the presence of the organisms in the digestive canal, one may easily suppose that these changes must also exist in rabbits because the organisms were detected from the digestive canal in high percentages.

As described above, the author confirmed that the rabbit has susceptibility rather similar to that of the natural host, especially of foals, to this organism.

The behavior of mouse in reaction to this organism which was described by OCHI, SOEKAWA, HIRATO and co-workers[8,9], and TOBA, of guinea-pig by HOSOYA, and of hamster by SHIMOJO & NOMURA and NOMURA, was considerably severer than that of the horse, therefore these animals may not be suitable for experimental studies with this organism.

From the above described data, it may safely be said that rabbits are the most suitable animal to use as substitutes for the horse especially of foals against *S. abortivoequina*. However, one must be extremely careful not to employ rabbits affected with coccidium, because the percentages of such affected rabbits is surprisingly high.

SUMMARY

The behavior of the rabbit against infection with *Salmonella abortivoequina* was examined and discussed.

The data obtained are summarized as follows:

1. Total 32 rabbits were perorally inoculated with the organisms ranging in number from 50 to 25,000 million.

2. Almost all rabbits revealed the symptoms of the infection. The severity of infection may not coincide with the numder of the organisms inoculated, but depend upon the individual susceptibilities.

3. The main symptoms of the disease in rabbits were rise of temperature (23/32=71.9%) and decrease of body weight (28/32=87.5%).

4. The antibodies generally began to rise after the 4th day following inoculation, and tended to appear in the following delaying order: hemagglutinin, O- and H-agglutinin, precipitin, and CF antibody.

5. After 8 weeks or later the antibodies began to fall. Among them, hemagglutinin and precipitin fell earlier, and CF antibody fell a little later or simultaneously with formers but it persisted in exhibiting a low titer for long period. O- and H-agglutinin fell gradually but in all cases they did not completely disappear during the observation period.

6. It seems to be probable that the rabbits which continued to show high precipitin titers, usually harbored the organisms.

7. The organism was detected most frequently from the mesenteric lymphnode and then in the following descending order: the spleen, submaxillary lymphnode and digestive canal.

8. The increase and decrease of the leucocyte number differ from each other, but it may be said that when febrile attack appears, leucopenia will occur and that when temperature becomes normal, it will turn to leucocytosis.

9. The "shift-to-the-left" of neutrophile leucocytes was evident in almost all cases.

10. Macroscopically, the enlargement of the mesenteric lymphnode and the spleen was observed in parallel with the detection of the organisms.

11. From the above stated results, it may safely be said that the rabbit has a susceptibility considerably similar to that of the horse, especially of foals, to the infection of *S. abortivoequina*.

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