On the Correlation between the Virulence of Brucella abortus and Their Growth in Sera of Several Animals

Tohoku Journal of Agricultural Research
Vol. 25, No. 2, November 1974
Printed in Japan

On the Correlation between the Virulence of *Brucella abortus* and Their Growth in Sera of Several Animals

Masayuki NAKAMURA and Masanori KATSUNO

Department of Animal Science, Faculty of Agriculture, Tohoku University, Sendai, Japan

(Received, November 29, 1974)

The growth of *Brucella abortus* in normal sera under 10 per cent CO_2 was observed. Avirulent *Brucella abortus* strain 19 was very sensitive to the bactericidal action of sera from normal cattle, guinea-pig, rabbit, sheep and human, but virulent strain 544 multiplied in these sera. Since the bactericidal factor in bovine serum was found to be heat-labile, relatively acid-labile and removable by an absorption with heat-killed brucella organisms, it seems that the action occurs in cooperation with normal antibody and complement. In these animals, this humoral factor should be related to the virulence of the two strains of *Br. abortus*.

On the other hand, in such animals as mouse, rat, hamster, dog and pig of which sera had not bactericidal action to strain 19, there may be something other than the humoral one to depress the organism.

There have been many studies on the virulence of *Br. abortus* (1, 2) and it is generally known that there are many smooth strains having different virulences. However, the essence of the virulent or the avirulent has not been clear. Olsen (3) reported that oxidative rates of glutamic acid were correlative with the virulences of strains of *Br. abortus*. FitzGeorge *et al.* (4) demonstrated that there was a correlation between the virulences of the strains for guinea-pigs and their sensitivities to hydrogen peroxide. Williams *et al.* (5) found another correlation between the virulence and growth enhancement by erythritol. And then Smith and FitzGeorge (6) reported that the ability of virulent *Br. abortus* to survive and multiply intracellularly was due to a cell wall substance which interferes with the intracellular bactericidal mechanisms of phagocytes.

It is also known that some strains of *Br. abortus* were sensitive to serum bactericidal action (7, 8). However, any correlation between the sensitivity and the virulence of the strains has not been clear. In this paper, the correlation was observed on sera of several species of animals and two strains, virulent and avirulent, of *Br. abortus*.

A part of this article was presented in the 75th Meeting of the Japanese Society of Veterinary Science held in April 1973 in Tokyo.

Materials and Methods

Brucella Strains

Br. abortus strain 544 (st. 544) and strain 19 (st. 19) were used. It is well known that the former is virulent and requires an increased CO_2 tension for its growth and the latter is avirulent and grows in an usual aerobic condition.

Immediately before the experiments, the strains which had been kept on Tripticase Soy Agar (BBL) slant were passed through mice three times. Freshly isolated organisms from the mouse were incubated on Tripticase Soy Agar plates at 37°C for three days. Then, the colonies were observed by the oblique light method (9) to confirm that they were the smooth type. The smooth organisms were harvested, washed and appropriately suspended with PBS (pH 7.2) for each purpose of the experiments.

Determination of the Virulence

Two groups of female Hartely guinea-pigs weighing 200–300 g were subcutaneously inoculated with 0.5 ml of the bacterial suspension containing 3.0×10^9 cells of st. 544 or 5.6×10^9 cells of st. 19. Two animals from the both groups were sacrified successively at some intervals during from the first day to 120th day of post-inoculation. Spleen, liver, kidney, uterus and inguinal lymph node were aseptically removed and homogenized quantitatively with PBS for counting the viable brucella cells. The viable count was performed by ordinary method with or without CO_2 according to the nature of the strains.

Female dd mice at five weeks of age were also inoculated with the suspension containing 9.5×10^8 cells of st. 544 or 6.4×10^8 cells of st. 19. Each five animals were killed weekly up to the 5th week and the viable count of spleen and liver was done in the same manner as in the case of guinea-pigs.

Observation on the Growth of Brucella in Sera

Sera from ten species of animals, as shown in Table 1, were prepared aseptically and used without inactivation. A half ml of the bacterial suspensions involving about 10^6 of viable organisms was added into the test tubes containing 0.5 ml of each serum. These tubes were immediately kept under the 10 per cent CO_2 at 37° C for 72 hours. During the incubation, viable cells were counted out at scheduled intervals.

Absorption of Bactericidal Factor

Fifty mg of st. 544 or st. 19 previously heated at 100°C for 30 minutes was added respectively into 0.5 ml of serum. After standing for 1 hour at 4°C, the cells were removed by centrifugation. Bactericidal activity of the supernate was determined.

Results

Virulence of Employed Strains

Different patterns of multiplication of the two brucella strains in guinea-pigs inoculated with 10^9 cells are shown in Fig. 1. In spleen and inguinal lymph node, st. 544 gave many bacterial counts from the first day to the 30th day of the experiment, which were about 10^7 per gram of the tissue. And then, it declined gradually but was still found at the 120th day. On the other hand, st. 19 dissapeared rapidly within 15 days though it was found in similar value to st. 544 at the first day. Similar patterns of the two strains were also obtained in liver, though the values were lower than in the above organs. In uterus and kidney, the number of bacteria was always under 10^4 and st. 544 was recovered until the 10th day but st. 19 was not found before the 7th day.

The bacterial growth in mcie inoculated with 10^8 cells is shown in Fig. 2. In spleen, the number of st. 544 was always higher than 10^3 during the first 4 weeks after inoculation and the organisms still recovered at the 5th week. Organisms of

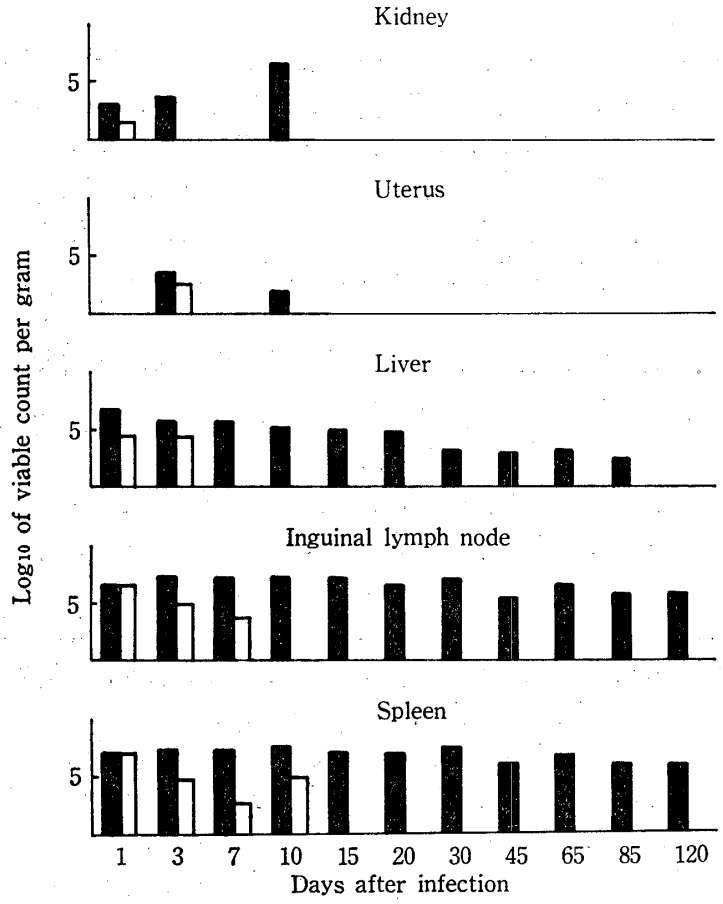

FIG. 1. Fate of *Br. abortus* st. 544 and st. 19 in organs of guinea-pigs. Guinea-pigs were inoculated subcutaneously with 3.0×10^9 cells of st. 544 or 5.6×10^9 cells of st. 19. Symbols indicate the geometrical mean of two animals. (■: st. 544, □: st. 19)

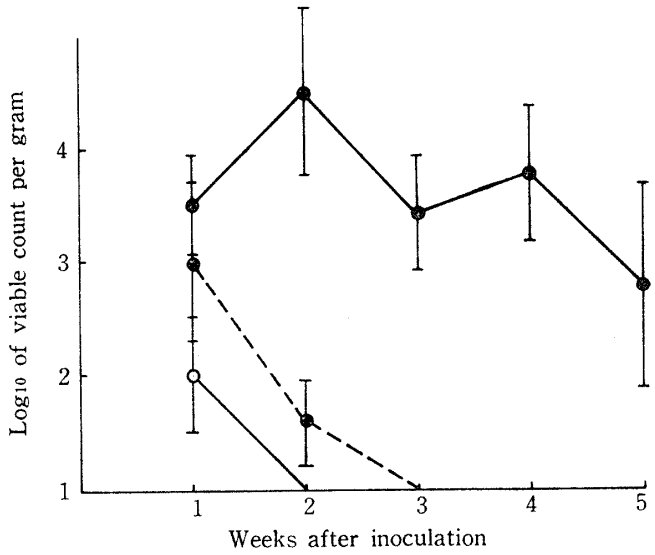

FIG. 2. Fate of *Br. aboruts* st. 544 and st. 19 in spleen and liver of mice. Mice were inoculated with 9.5×10^8 cells of st. 544 or 6.4×10^8 cells of st. 19. Symbols indicate the geometrical mean and S.D. of five animals. (——: st. 544, – – –: st. 19, ●: spleen, ○: liver)

st. 19 being 10^3 in number at the first week decreased rapidly until they could not be recovered at the third week. In liver, st. 544 was recovered at the first week but st. 19 disappeared within one week.

From these results it is said that st. 544 lived more and longer than st. 19 in guinea-pig and mouse. Thus, it is confirmed that st. 544 used in these experiments had high virulence for guinea-pig and mouse, while st. 19 was low virulent.

Growth of St. 544 and St. 19 in Sera of Guinea-pig and Cattle

The growth of the two strains in normal sera of guinea-pig and cattle was observed. The results are shown in Fig. 3. Virulent st. 544 multiplied gradually in guinea-pig serum, though a slight decrease of viable count was observed in the first 6 hours. At the same time, avirulent st. 19 remarkably decreased during the first 48 hours. These facts show that the virulent strain which can easily multiply in guinea-pig can also grow in its serum and the avirulent strain which can hardly multiply at all in the animal does not grow well in the serum. It seems that st. 19 affected by serum bactericidal action.

Also, the effects of bovine serum on the two strains were investigated, as it was thoutht that one was virulent to cattle and another was avirulent. The results were as same as in the case of serum of guinea-pig.

Thus, it was found that there was a correlation between the virulence of the *Br. abortus* to the guinea-pig or cattle and the *in vitro* growth in their sera.

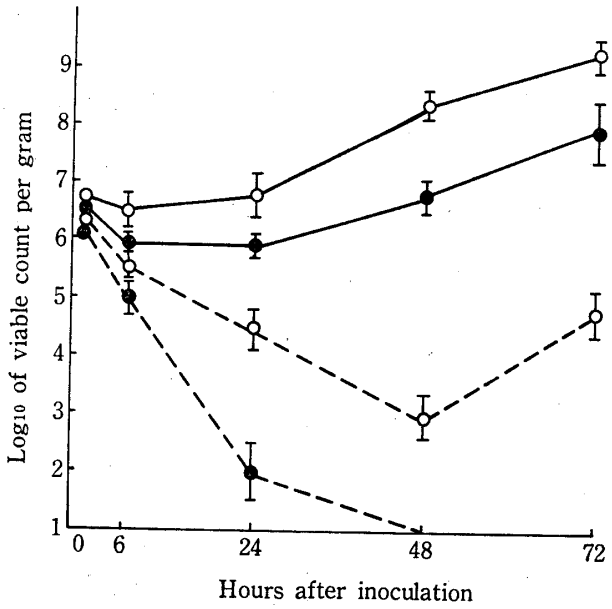

FIG. 3. *In vitro* growth of *Br. abortus* st. 544 and st. 19 in bovine and guinea-pig sera. Symbols indicate the geometrical mean and S.D. of five sera kept at 37°C under the 10% CO_2. (——: st. 544, – – –: st. 19, ●: cattle, ○: guinea-pig)

Effects of Normal Sera of Various Animals on St. 544 and St. 19

As it was found that the bovine and guinea-pig sera were harmful for the growth of st. 19, the following experiments were carried out to determine whether the same effect was found in sera of the other animals. The results are shown in Table 1.

TABLE 1. *In vitro Growth of Br. abortus st. 544 and st. 19 in Sera of Various Animals*

Animals	Number of viable cells		Animals	Number of viable cells	
	st. 544	st. 19		st. 544	st. 19
Guinea-pig	8.38±0.28	2.93±0.36	Mouse	9.83±0.58	9.96±0.49
Rabbit	6.54±0.24	2.28±0.16	Rat	9.83±0.18	9.78±0.21
Sheep	4.52±0.50	<1.00	Hamster	9.76±0.36	9.63±0.28
Cattle	6.86±0.25	<1.00	Dog	8.58±0.11	8.04±0.32
Human	8.83±0.60	<1.00	Pig	8.72±0.05	9.01±0.40

Viable cells ranging from 2.0×10^6 to 9.6×10^6 of *Br. abortus* st. 544 or st. 19 were inoculated into serum and kept at 37°C under the 10% CO_2 for 48 hours. Values are presented with $\log_{10}$ of viable count and indicate the geometrical mean and S.D. of five animals sera.

In every serum, the viable count of st. 544 detected at 48 hours after the inoculation was not lower than that of the initial numbers, except in the case of sheep serum, where the organisms relatively decreased. At 48 hours, st. 19 remarkably decreased in sera of guinea-pig, rabbit, cattle and human, though it

showed similar growth in sera of mouse, rat, hamster, dog and pig as compared with st. 544.

Thus, it was known that one group of animals such as guinea-pig, rabbit and so on had the bactericidal action for st. 19 in their sera but another group involving mouse, rat and so on did not have such action. The serum bactericidal activity for st. 19 was stronger in cattle, sheep and human than in guinea-pig and rabbit.

Characteristics of Anti-st. 19 Factor

As above mentioned, there was a bactericidal factor for st. 19 in the sera of several animals. However the characteristics of the factor has not been clear. The following experiments were done to detect the nature of the factor in bovine serum, where activity was high.

In the sera previously heated at 56°C for 5 to 30 minutes, st. 19 multiplied similar to st. 544, though the former was affected harmfully by intact serum, as shown in Table 2. The fact shows that the bactericidal factor is extreamly heat-labile.

TABLE 2. *Growth of Br. aboruts st. 544 and st. 19 in Bovine Serum Previously Heated at 56°C*

Time of pretreatment	Number of viable cells	
	st. 544	st. 19
0 min.	6.63	2.32
5	7.52	7.28
10	7.56	7.26
20	7.83	7.78
30	7.31	7.01

6.54×10^6 cells of st. 544 or 6.83×10^6 cells of st. 19 were inoculated into serum. Values are presented with $\log_{10}$ of viable count after 48 hours at 37°C under the 10% CO_2.

Table 3 shows the stability of the factor in acid and alkaline circumstance. After the sera were adjusted to pH 3–9 with diluted HCl and NaOH and kept 1 hour at room temperature, they were readjusted to pH 7.2 and then inoculated with brucella organisms. As a result, the bactericidal factor for st. 19 was observed at pH ranging from 5 to 9 but not at pH 3 and 4. The factor seems to be unstable to acid.

Optimal temperature of the bactericidal effect on st. 19 is shown in Table 4. When st. 19 incubated with normal bovine serum at 25 to 37°C for 48 hours, the viable cells decreased. But such effect was not seen at 4 to 15°C. Therefore, the serum bactericidal activity is temperature-dependent.

Effects of absorption with heat-killed brucella organisms on the serum activity are shown in Table 5. The activity on st. 19 was completely removed

TABLE 3. *Growth of Br. abortus st. 544 and st. 19 in Bovine Serum Previously Treated with Acid and Alkali*

pH of pretreatment	Number of viable cells	
	st. 544	st. 19
Untreated	7.56	5.48
3	7.68	7.63
4	8.15	7.68
5	8.13	5.22
6	7.83	5.51
7	7.78	5.46
8	7.67	5.41
9	7.51	5.55

6.32×10^6 cells of st. 544 or 6.85×10^6 cells of st. 19 were inoculated into serum readjusted at pH 7.2. Values are presented with $\log_{10}$ of viable count after 48 hours at 37°C under the 10% CO_2.

TABLE 4. *Effect of Incubation Temperature on Growth of Br. abortus st. 544 and st. 19 in Bovine Serum*

Incubation temperature	Number of viable cells	
	st. 544	st. 19
4°C	6.23	5.82
15	6.31	5.85
25	6.28	2.65
30	6.31	2.76
37	6.56	2.03

8.63×10^5 cells of st. 544 or 9.60×10^5 cells of st. 19 were inoculated into serum. Values are presented with $\log_{10}$ of viable count after 48 hours at indicated temperatures under the 10% CO_2.

TABLE 5. *Growth of Br. abortus st. 544 and st. 19 in Bovine Serum Absorbed with Heat-killed Brucellae*

Absorbed with	Number of viable cells	
	st. 544	st. 19
None	7.01	2.06
st. 19	8.76	8.83
st. 544	8.89	8.68

6.78×10^6 cells of st. 544 or 6.38×10^6 cells of st. 19 were inoculated into absorbed serum. Values are presented with $\log_{10}$ of viable count after 48 hours at 37°C under the 10% CO_2.

from the serum by the pretreatment with st. 19 at 4°C. Also, similar result was obtained with st. 544. These facts indicate that the bactericidal factor combines with brucella organisms, regardless of their virulence.

Discussion

Avirulent strain 19 of *Br. abortus* was harmfully affected in normal sera of cattle, guinea-pig, rabbit, sheep and human (Table 1). Joos and Hall (8) observed the bactericidal action of rabbit serum to st. 19. Huddleson *et al.* (5) also reported that bovine serum and plasma were bactericidal for *Br. abortus* which is thought to be CO_2-independent avirulent strain judging from its culture condition, and they said that the rough phase of Br. abortus was more sensitive to serum bactericidal action than the smooth phase. In contrast with st. 19, virulent st. 544 multiplied in these sera (Table 1).

From these facts, it is thought that the humoral factor plays an important role in defference mechanisms of these animals against st. 19 of *Br. abortus*. In other words, the virulence of the two strains of *Br. abortus* may be related to the sensitivities to the bactericidal factor in the sera of them.

On the other hand, sera of mouse, rat, hamster, dog and pig did not show such bactericidal action (Table 1). As st. 19 was also avirulent for mouse (Fig. 2), these animals may have something to depress the organisms other than the humoral factor. It may be cellular.

The bactericidal factor for st. 19, being in bovine serum, was absorbed with heat-killed cells of avirulent st. 19 or virulent st. 544 (Table 5). Nevertheless, the virulent strain was resistant to this factor. These facts suggest that there may be some structural difference between the two strains.

The absorption occured at 4°C (Table 5), though the bactericidal effect did not appear at the low temperature (Table 4). The bactericidal action may be related to physiological condition of *Br. abortus*. This is supported by observations that sensitivity to the bactericidal action of bovine serum was different between slant-grown brucella organisms and monocyte-grown ones (10).

From another point of view, it seems that the bactericidal action occurs in cooperation with at least two agents, one of which can combine with bacterial cells at low temperature but another needs moderate temperature for its reaction. Also, these bactericidal agents were heat-labile and relatively acid-labile. Putting these results together, it may be thought that the agents are normal antibody and complement.

References

1) Wilson, J.B., and Dasinger, B.L., *Ann. N.Y. Acad. Sci.*, **88**, 1155 (1960)
2) Smith, H., *Bacteriol. Rev.*, **32**, 164 (1968)

3) Olsen, C.B., The aerobic metabolism of *Brucella abortus*. M.S. Thesis. Univ. Wis. (1951)
4) FitzGeorge, R.B., Keppie, J., and Smith, H., *J. Path. Bact.*, **89**, 745 (1964)
5) Williams, A.E., Keppie, J., and Smith, H., *J. Gen. Microbiol.*, **37**, 285 (1964)
6) Smith, H., and FitzGeorge, R.B., *Brit. J. Exp. Path.*, **45**, 174 (1964)
7) Huddleson, F., Wood, E.E., Cressman, A.R., and Bennet, G.R., *J. Bacteriol.*, **50**, 261 (1945)
8) Joos, R.W., and Hall, W.H., *J. Bacteriol.*, **96**, 881 (1968)
9) Henry, B.S., *J. infect. Dis.*, **52**, 374 (1933)
10) Stinebring, W.R., Brawn, W., and Pomales-Lebrón, A., *Ann. N.Y. Acad. Sci.*, **88**. 1230 (1960)