Spontaneous *Brucella canis* Infection in Beagles: Bacteriological and Serological Studies

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Abstract. Fifteen beagles, 13 males and 2 females, from a breeder colony suspected of *Brucella canis* (*B. canis*) infection were autopsied, and isolation of *B. canis* from various organs and detection of anti-*B. canis* agglutinin in their sera were attempted. *B. canis* was isolated from the spleen, lymph nodes, prostate and epididymis at a high frequency and from blood, testis and urinary bladder of some cases. Five of 15 dogs having been treated with tetracycline were shown to harbour smaller number of the organisms than the non-treated ones. Most of the sera showed high agglutinin titres to *B. canis*, and the presence of incomplete antibody was confirmed in some sera showing only feeble agglutination. Treatment with cortisone or antilymphocytic serum failed to produce recurrent disease in 12 persistently infected males.

An outbreak of abortions in beagles occurred during a period from December 1971 to August 1972 in a breeding colony of beagles in Shizuoka Prefecture [4]. Some of breeder males showed swelling of the testis or epididymis about one month before the initiation of the outbreak of abortion. In July 1972, *Brucella*-like coccobacilli were isolated from one of aborted fetuses of this colony by Dr. Azuma, National Institute of Animal Health, Tokyo (personal communication), and thus, *B. canis* infection [4], a disease first described in U.S.A. [1, 3, 6, 8] was suspected for this prevailing of abortion in beagles. Sera taken in June 1972 from dogs in this colony were tested by Dr. Carmichael at the Cornell University, Ithaca, for examining anti-*B. canis* agglutinin, and they were shown to be positive. An eradication program

was then started through checking infected animals by detecting *B. canis* in circulating blood or serum agglutinin, and the dogs diagnosed as infected were separated from non-infected ones. Then, incidence of abortion ceased in this colony after September 1972 [14].

Some of these infected and isolated beagles were autopsied by the present authors during a period from August 1972 to April 1973. Further, in February 1973, some other infected dogs were served for an experiment to see the effect of immunosuppressive agents upon provocation of long lasting infection of *B. canis*. This report deals with bacteriological and serological findings in these examinations.

Materials and Methods

Beagles: Thirteen male and 2 female adults of

ビーグル犬における *Brucella canis* 自然感染—細菌学および血清学的検索： 上田雄幹・曲淵輝夫・三枝順三・浦野 徹・伊藤喜久治・木内吉寛・藤原公策（東京大学医科学研究所獣医学研究部）

Table 1. History of dogs autopsied

Date of autopsy	No.	Sex	Tetra-cycline	Age at necropsy	First clinical signs	Blood culture before examination****			
						28 Jul.	28 Aug.	12 Oct.	12 Nov.
17 Aug. 1972	ZD-14	M	—	1Y 10M	Atrophy of testis (May '71) (Abortion in partner mated in Feb. '72)	+			
	QW-12	M	—	2Y 5M	Swelling of testis (Nov. '71)	+			
	FU-23	F	—	2Y	Abortion (Apr. '72)	+			
	QE-11	F	i.v.*	1Y 11M	Abortion (May '72)	+			
19 Sep. 1972	LG-23	M	—	2Y 4M	Swelling of testis (Nov. '71) (Abortion in partner mated in Oct. '71)	C	ND		
	QP-16	M	—	2Y 8M	(Abortion in partner mated in Feb. '72)	C	+		
	ZD-12	M	i.v.	1Y 11M	(Abortion in partner mated in Oct. '71)	+	ND		
	NA-22	M	i.v.	1Y 8M	Swelling of testis (Nov. '71)	+	ND		
28 Nov. 1972	70-253	M	—	2Y 2M	?	+	ND	—	ND
	70-255	M	—	2Y 2M	?	+	ND	—	ND
	70-243	M	oral**	2Y 3M	(Abortion in partner mated in Nov. '71)	—	ND	—	ND
25 Apr. 1973	71-1	M	i.v.+oral	1Y 11M	?	+	ND	—	ND
	BK-72	M	fed***	2Y 2M	Swelling of testis (Nov. '71) (Abortion in partner mated in Jun. '71)	C	+	—	ND
	BZ-62	M	fed	3Y 2M	(Abortion in partner mated in Dec. '71)	—	—	+	—
	IS-22	M	fed	3Y 2M	Swelling of testis (Nov. '71) (Abortion in partner mated in Feb. '72)	—	—	+	+

Remarks.

*: Tetracycline 20mg/kg daily i.v. for 3 weeks (Jul.-Aug.)

**: Tetracycline 30-40mg/kg daily per os for 3 weeks (Oct.-Nov.)

***: Tetracycline mixed in food, ca. 30mg/kg for 3 weeks in Jan.

****: See reference [14] for the method of blood culture, +: positive culture, -: negative culture, C: contamination, ND: not done.

age about 2 to 3 years were served for autopsy, 3 to 4 dogs in each time, in August, September, November 1972 and April 1973. Some of them had been treated with tetracycline. Age at autopsy of these beagles are listed in Table 1 together with the history of clinical and bacteriological examinations and treatments made before the autopsy. Although exact time of infection remained unknown, probable signs of infection were able to trace back to November 1971 in some dogs by referring their breeding records. The most well-defined signs were abnormalities of genitalis, such as enlargement or atrophy of the testis or epididymis, and abortion in the females. There were

some males whose only recorded sign of the infection was abortion having occurred in their partner females, as shown in parenthesis in Table 1. Blood cultures made before the autopsy of these dogs revealed that almost all of them had bacteremia at least once in their history. Dogs used for provocation experiment were 12 males aged from 8 months to 2 years at the time of experiment, February 1973. They were evidenced to be infected mostly by blood culture, and in some cases by high levels of serum agglutinating titre. All of them had been treated with tetracycline or streptomycin [14]. Dogs showing decreasing serum titre by the end of 1972, and negative blood culture

after January 1973 were subjected to the experiment.

Isolation of *Brucella* from the blood and organs: Two to 3 ml of blood was taken aseptically from the cervical vein and was inoculated into 200 ml of trypticase soy broth (BBL) without adding anticoagulant. They were incubated at 37°C for 5 days. After 1, 3 and 5-day's incubation loopfull samples from the broth cultures were streaked onto tryptic soy agar (TSA) (Difco) plates, and the plates were incubated for 3 days aerobically. On the other hand, the following organs were subjected to isolation of *Brucella* organisms, liver, spleen, kidney, lung, iliac and popliteal lymph nodes, urinary bladder and bone marrow, and in addition, testis, epididymis and prostate for males, and uterus, ovary and vagina for females. For isolating organisms from the organs, cut-surface of pieces of aseptically removed organs except for the bone marrow were streaked on TSA plates. In case of the bone marrow, a loopful sample from the humoral marrow was streaked on TSA agar plate. These plates were incubated at 37°C for 7 days aerobically. Some of suspected colonies developed were isolated and were screened by slide agglutination test with mouse or rabbit antiserum to *B. canis*. Mouse serum showing an agglutinin titer of 1:400 was a pool of sera from mice immunized with formalin-killed *B. canis* strain QE-11 (one of the isolates in the present examination) in Freund's complete adjuvant. Anti-*B. canis* rabbit serum was prepared by immunizing with heat-killed (60°C for 60 min) *B. canis* strain RM 666 in Freund's complete adjuvant. And then, the following biological characteristics were examined for identifying the isolates: Gram stain (—), acriflavin agglutination (+), glucose (—), lactose (—), sucrose (—), mannitol (—), maltose (—), gelatin (—), urease (+), nitrate (+), indol (—), motility (—), VP (—), MR (—), catalase (+), citrate (—) and Litmus milk (—). *B. canis* strain RM 666 was employed as a reference strain. Further bacteriological identification for *B. canis* was made by Dr. W. J. B. Morgan in Weybridge, after sending representative six isolates through Japan Brucellosis Center, and all the six isolates were revealed to have characteristics of *B. canis* (personal communication).

Serum agglutination test: An antigen to *B. canis*, strain RM 666 prepared according to Carmichael [2] was obtained by the courtesy of Dr. Ghoda at the Kitasato Institute, Tokyo. The method for the antigen preparation informed for Dr. Ghoda was as follows.

The organisms grown on TSA agar after incuba-

tion for 48 hrs were harvested in phosphate-buffered saline (PBS) pH 7.2, and after filtration through gauze, they were heated at 60°C for 60 min. After washing twice with PBS the killed organisms were resuspended in PBS. Merthiolate was added at 0.01%, and the antigen was kept at 4°C. Before use, the antigen was diluted with PBS to give an optical density of 0.20 with 420 nm filter using Hitachi Spectrophotometer.

Two-fold dilutions of test sera starting usually at 1:25 were made, and one ml of the antigen suspension was added to 0.5 ml of serum dilutions. The mixtures were incubated at 50 to 52°C and expressed by a dilution of serum before adding the antigen suspension giving a complete agglutination. Techniques for blocking as well as Coombs' tests will be described with the experimental results.

Results

Isolation of *B. canis* from the blood and various organs: As shown in Table 2, *B. canis* was isolated from 13 of 15 beagles examined. Among organs examined, a relatively high positivity exceeding 50% was observed in the spleen, iliac and popliteal lymph nodes, epididymis and prostate and 30 to 50% of the blood, testis and urinary bladder samples were also positive. On the bone marrow, culture was obtained in one of 8 cases examined. On one of 2 females examined, which had aborted 3 months previously, the organisms were isolated from the non-pregnant uterus.

From 5 dogs which had received intravenous or peroral treatment of tetracycline, no or very few organisms were isolated from the organs. However, effects of the antibiotic treatment was not obvious in 3 dogs having received tetracycline in diet, when examined at killing in April 1973.

Serology: Results of the agglutination test are shown also in Table 2. High titers of 1:100 to 1:400 were obtained in all the dogs autopsied in September or November in 1972 and April in 1973. However, 4 dogs autopsied in August 1972 showed negative or feeble reaction at 1:25

Table 2. Isolation of *B. canis* from various organs

Date of autopsy	Dog	Sex	Tetra-cycline treatment*	Organ															No. positive No. organs examined	Agglutinin titre
				Blood	Liver	Spleen	Kidney	Lung	Iliac lymphnode	Popliteal lymphnode	Testis	Epididymis	Prostate	Uterus	Ovary	Vagina	Urinary bladder	Bone marrow		
17 Aug. 1972	ZD-14	male	none	**	***	4	1	1	4	0	3	2	2	.	.	.	1	.	10/11	****
	QW-12	male	none	+	1	3	0	0	3	3	1	3	0	.	.	.	0	.	7/11	25>
	FU-23	female	none	+	0	1	0	0	1	1	.	.	.	1	0	0	0	.	5/11	25>
	QE-11	female	i.v.	+	0	1	0	0	0	0	.	.	.	0	0	0	0	.	2/11	25>
19 Sep. 1972	LG-24	male	none	C	0	1	0	1	2	0	1	1	2	.	.	.	2	2	8/12	200
	QP-16	male	none	+	2	3	0	2	1	2	0	2	2	.	.	.	0	0	8/12	200
	ZD-12	male	i.v.	C	0	0	0	0	0	0	0	0	0	.	.	.	0	0	0/12	200
	NA-22	male	i.v.	0	0	0	0	0	1	0	0	1	0	.	.	.	1	0	3/12	200
28 Nov. 1972	70-253	male	none	C	0	3	0	0	3	2	0	0	0	.	.	.	0	0	3/12	400
	70-255	male	none	C	0	1	0	0	1	1	0	0	0	.	.	.	0	0	3/12	200
	70-243	male	per os	C	0	0	0	0	0	0	0	0	0	.	.	.	1	0	2/12	400
	71-1	male	i.v. + per os	C	0	0	0	0	0	0	0	0	0	.	.	.	0	0	0/12	100
25 Apr. 1973	BK-72	male	fed	0	0	1	0	0	2	1	2	2	3	.	.	.	0	.	6/11	100
	BZ-62	male	fed	0	1	2	1	1	2	1	1	1	3	.	.	.	2	.	10/11	200
	IS-22	male	fed	0	1	3	1	1	3	3	3	3	3	.	.	.	1	.	10/11	400
No. positive cases				5	5	11	3	5	11	8	6	8	7	1	0	0	6	1		
No. examined				/	/	/	/	/	/	/	/	/	/	/	/	/	/	/		
				9	15	15	15	11	15	15	13	13	13	2	2	2	15	8		

Remarks.

* See the foot-notes on Table 1.

** Positive in broth culture.

*** Numbers indicate the degree of bacterial growth per plate as follows.

4: Confluent growth.

3: More than 100 colonies.

2: 10-100 colonies.

1: Less than 10 colonies.

0: No growth.

C: Contamination.

**** Reciprocal of the highest dilution showing positive agglutination.

to 1:400 (Table 3). A high titer was obtained in 2 tetracycline-treated dogs from which the organisms was not detectable.

To see whether the feeble agglutination with the sera of 4 dogs killed in August 1972 is specific or not, a blocking test was performed. After reacting the routine agglutination 0.025 ml of 1:5 dilution of anti-*B. canis* mouse serum described above was added to each reacting tube. The mixtures were incubated another 48 hrs at 50°C for the final reading. Though not shown

in the table, a positive reaction was seen in dogs ZD-14 and QW-12 at 1:25 and 1:50, respectively, suggesting the presence of incomplete antibody.

With the some sera including ZD-14 and QW-12, Coombs' test was carried out. A primary agglutination was made employing 1.5 ml of serum dilutions of test sera and 3 ml of antigen suspensions. After reading the results of agglutination, the serum-antigen mixtures to be examined were centrifuged and washed 3 times with PBS. The

Table 3. Agglutination and Coombs' tests on some sera harvested at autopsy

Primary agglutination

Serum	Serum dilution						
	1 : 12.5	1 : 25	1 : 50	1 : 100	1 : 200	1 : 400	1 : 800
ZD-14	0*, **	0**	0	0	0	0**	0
QW-12	0**	0**	1	1	1	0**	0
FU-23	2	1	1	1	0	0**	0
QE-11	2	1	1	1	0	0**	0
LG-24	3	3	3	3	2	0**	0
QP-16	3	3	3	3	3	0**	0
ZD-12	3	3	3	3	2	0**	0
NA-22	3	3	3	3	2	0**	0

Remarks.

*: Numbers indicate the degree of agglutination as follows :

0—No agglutination.

1—Feeble agglutination.

2—Complete agglutination with slightly turbid supernatant.

3—Complete agglutination with clear supernatant.

**: These tubes were used for the Coombs' test.

Secondary agglutination with anti-dog gammaglobulins

Serum	Primary serum dilution	Agglutination with		
		RADS*	RNRS**	PBS
ZD-14	1 : 12.5	+	—	—
	1 : 25	+	—	—
	1 : 400	+	—	—
QW-12	1 : 12.5	+	—	—
	1 : 25	+	—	—
	1 : 400	+	—	—
FU-23	1 : 400	+	—	—
QE-11	1 : 400	+	—	—
LG-24	1 : 400	—	—	—
QP-16	1 : 400	—	—	—
ZD-12	1 : 400	—	—	—
NA-22	1 : 400	—	—	—

Remarks.

*: Rabbit antiserum to dog serum.

**: Normal rabbit serum.

final sediment was resuspended with 3 ml of PBS and distributed to 3 tubes, one ml each, to which were added 0.5 ml of a 1 : 20 dilution of rabbit antiserum to dog gammaglobulin equivalent to 4 units of precipitating titre against whole dog-serum. The anti-dog gammaglobulin serum was a gift from Dr. A. Hasegawa, Faculty of Agriculture, The University of Tokyo. As

controls, normal rabbit serum (1 : 20) or PBS was added in place of antiserum to dog-gammaglobulin. The mixtures were incubated at 37°C for 24 hrs and agglutination was read. As shown in Table 3, sera from ZD-14 and QW-12 showed positive reaction at 1 : 12.5, 1 : 25 and 1 : 400. Somewhat weak but obvious agglutination was seen also in sera of FU-23 and QE-11

Table 4. Isolation of *B. canis* after administration of cortisone or ALS

Group	Dog	Organs										
		Blood	Liver	Spleen	Kidney	Lung	Iliac lymphnode	Popliteal lymphnode	Urinary bladder	Testis	Epididymis	Prostate
A	AT2-M2	0*	0	0	0	0	2	0	0	0	3	1
	AQ2-M2	0	0	0	0	0	0	0	0	0	0	0
	CG1-M1	0	0	0	0	0	0	0	0	0	0	0
	LG-22	0	0	0	0	0	0	0	0	0	0	0
B	AT2-M1	0	0	0	0	0	0	0	0	0	0	0
	BB2-M1	0	0	0	0	0	0	0	0	0	0	1
	DB1-M3	0	0	0	0	0	0	0	0	0	0	0
	71 - 9	0	0	0	0	0	2	1	0	0	0	2
C	DF1-M2	0	0	0	0	0	0	0	0	0	0	0
	DE1-M1	0	0	0	0	0	0	0	0	0	0	0
	CD1-M1	0	0	0	0	0	0	0	0	0	0	1
	LHO-22	0	0	0	0	0	0	0	0	0	0	0

Remarks.

*: See the foot-note of Table 2.

at 1:400. Although anti-dog globulin rabbit serum (RADS) and control normal rabbit serum (NRS) had not been absorbed with the *B. canis* antigen, they did not agglutinate the antigen at least at a dilution of 1:20.

To see resistance of the agglutinin to reducing activity of 2-mercaptoethanol (2-ME), sera to be tested were mixed with equal volumes of 0.2M 2-ME and incubated at 37°C for 1 hr. Thereafter, serial 2-fold dilutions of the sera were made without dialysis and ordinary agglutination test was performed. Practically, no reduction was seen with all the sera showing positive agglutination.

Effect of cortisone and antilymphocyte serum (ALS) on subsided infection: Since it is assumed that the dogs treated with antibiotics still harbour a small number of the organisms in any organs, a provocation was attempted by administration of immunosuppressing agents. Twelve male dogs diagnosed as infected by positive blood culture or by highly elevated serum agglu-

tinin titre for *B. canis* infection and had been treated with antibiotics were divided into 3 groups: Group A for cortisone-treatment, Group B for ALS-treatment, and Group C for intact controls. Cortisone acetate (Merk, Banyu) at a dose of 25 mg per kg body weight was injected intramuscularly on Days 0, 2 and 4 of the experiment. Horse antiserum to beagle thoracic duct lymphocytes (ALS), a gift from Dr. M. Matsukura, Department of Organ Transplantation and Physiology of this Institute, was injected at a dose of one ml per kg body weight subcutaneously on Days 0 and 5.

Blood culture and measurement of agglutinin titre were made at intervals following the treatments, and all dogs were sacrificed on Day 47 of the experiment, and isolation of the organisms was carried out on various organs.

None of the dogs revealed bacteremia during the experimental period. As shown in Table 4, *B. canis* was isolated from 1 of 4 in Group A, 2 of 4 in Group B, and 1 of

4 in Group C, at autopsy. The numbers of colonies developed were very numerous in 2 from either Group A or B. There was no marked difference among the 3 groups in the pattern of agglutinin level following the treatments.

Discussion

The frequency of *B. canis* isolation from the organs and the anti-*B. canis* agglutinin titer in the sera were considerably high in the dogs examined, indicating that the abortion storm might have resulted from *B. canis* infection in this beagle dog colony. Since all the cases were spontaneous, it was difficult to ascertain the exact time of infection in individual cases. However, probable signs of infection could be estimated retrospectively by examining the breeding record of the dogs. According to this, it seemed that all the dogs examined had been infected with *B. canis* at least for 3 months, most likely for 6 to 12 months or even over. This estimation is not incompatible with the finding that, in almost all positive sera detected were 2-ME resistant agglutinin. It was reported already that 2-ME resistant agglutinin is produced in chronic *B. canis* infection [13].

Though it is probable, as mentioned above, that these dogs had been infected since a long time before the autopsy, the organism could be isolated frequently, especially in a high number from the lymphatic and reproductive organs. Persistence of the organisms for such a long period in infected dogs is consistent with the previous reports by other investigators [4, 9, 13] on spontaneously and experimentally infected dogs. The preferential distribution of the organism in the lymphatics as well as the reproductive organs seems to be characteristic of the infection of *B. canis* in dogs, and in this respect, the canine brucellosis due to

B. canis seems quite resemble to the classical three types of brucellosis in the susceptible hosts [11, 12].

Other than the lymphatic organs, the epididymis and prostate seem to be the most susceptible organs in males and, the infection in these organs has been shown to last for a long period like in the lymphatic organs. The infection in the genital organs might be responsible for the high incidence of organisms from the urinary bladders, but the incidence of isolation of the organism from the kidney was rather low. For the female genital organs, we can not comment much about because only 2 females were examined. The low positive culture rate, only 1 of 8, in the bone marrow might be due to a small amount of samples streaked on the plates. Blood culture was hampered much due to the contamination of the broth culture. However, attention has been attracted to the negative blood culture in spite of multiple isolations from the organs of dogs killed in April 1973. This finding seems indicate a necessity of frequent blood cultures for diagnosis.

It was confirmed, as reported by Carmichael [4], Moore [9] and van Hoozier [13], that a high titre of anti-*B. canis* agglutinin was persisting for long period in the infected dogs. Although sera from 4 dogs killed in August 1972 seemed to be unusual in this respect, they were shown to be positive in blocking as well as Coombs' tests, indicating the occurrence of incomplete antibodies which is known to occur occasionally in brucellosis of the goat, sheep and pig [11]. The reason why only these 4 dogs developed such antibodies remains unclear.

As indicated in the Materials and Methods, some of these dogs had been treated with an antibiotic, tetracycline. After the

treatment, the number of organisms in the organs greatly reduced in comparison to that of non-treated dogs. However, it seems unlikely that the antibiotic treatment did eliminate completely the organisms harboured in the organs. A recurrence of bacteremia has been reported after the termination of antibiotic treatment [7]. Thus, it was considered that the dogs given treatment of the antibiotics would harbour a small number of organisms in their organs. It was examined whether or not this persisting organisms can be propagated recurrently following administration of immunosuppressant. Results showed, however, that neither cortisone nor ALS resulted in a detectable provocation of the disease and affected on the level of serum antibody titres. Although there are some reports concerning the effect of cortisone [10] or ALS [5] on some infections due to facultative intracellular pathogens, positive effects of the immunosuppressant were observed only when the administration was made at an early stage of infection. On the contrary, persisting *B. canis* infection seems to be refractory to this kind of stimulation.

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