

イヌブルセラ症の伝播における尿の意義

誌名	日本獣医学雑誌 = The Japanese journal of veterinary science
ISSN	00215295
著者	芹川, 忠夫 村口, 武彦
巻/号	41巻6号
掲載ページ	p. 607-616
発行年月	1979年12月

Significance of Urine in Transmission of Canine Brucellosis

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(Received for publication March 22, 1979)

Abstract. When inoculated by various routes with approximately 10^8 (or 10^7) *Brucella canis* organisms, all adult dogs became infected. When dogs were inoculated with approximately 10^4 organisms by different routes, the positive rate of infection by the route of inoculation was as follows: 2/2 (No. infected/No. inoculated) (intravenous), 2/2 (subcutaneous), 2/4 (conjunctival), 0/4 (oral) and 0/3 (intravaginal). Five male dogs were inoculated orally with 3.2×10^8 *B. canis* organisms and examined weekly for urine. The organisms were first detected in their urine a few weeks after the onset of bacteremia. Then a high concentration of these organisms was usually excreted continuously. Five 4-week-old puppies born from a *B. canis*-free bitch were housed together with these infected males in an isolated facility for 18 weeks. One of them became infected. The transmission of brucellosis to the puppy seemed to have been caused by a contact with *B. canis*-contaminated urine.

Several groups of Japanese investigators isolated *Brucella canis* from dogs in some areas of Japan [9, 19, 21, 22, 26, 27]. At the present time, canine brucellosis appears to be prevalent in stray dogs all over the country. Natural spread seems to be caused most frequently by contact with infective vaginal discharge or aborted fetal and placental tissues [2, 4, 15-17, 20]. Venereal transmission has also been incriminated [4, 5, 15]. Furthermore, congenital infection has been reported [2, 5, 15, 16]. The organism has been isolated from the milk of infected females [15-17]. Although the presence of *B. canis* in the urine of infected dogs has been known in natural or experimental cases, whether the urine contaminated by *B. canis*, especially that excreted by male dogs, makes a significant mean of transmission has not been determined [13-16].

In order to determine this possibility, the authors examined first infective doses by

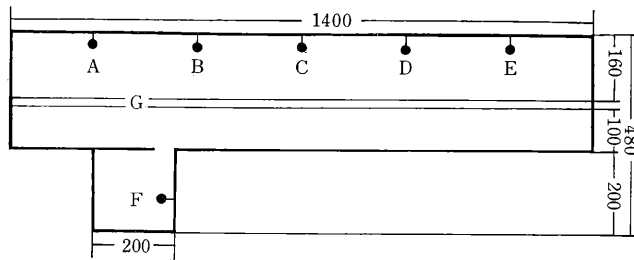
various routes, and then the concentration of the organism in the urine after experimental inoculation. Furthermore, a transmission experiment was made on 5 intact puppies housed together with infected males in an isolated facility. From the results obtained, the possibility of transmission of canine brucellosis by contaminated urine was proposed.

Materials and Methods

Dogs: A total of 25 adult male and female mongrels weighing 9 to 14 kg were bought from dog pounds. They were confirmed to be abacteremic and to have only low serum agglutinin titers to *B. canis* for about 1 month before use. Five male beagles about 2 years old weighing 10-12 kg were obtained from a *B. canis*-free colony. A non-infected mongrel bitch weighing about 12 kg with five 4-week-old puppies was also purchased.

Inoculum: A strain of *B. canis* isolated from the retropharyngeal lymph node of a spontaneously infected dog was used. The organism stored at -20°C was incubated in Trypticase soy broth (TSB, Eiken) at 37°C for 4 days. Then it was suspended in sterilized PBS (pH 6.2). Appropriate dilutions were

Fig. 1. Floor plan of the dog-housing facility used



Remarks.

- A-E: Hooks for chaining 5 male beagles (Nos. 1-5).
 F: A hook for chaining a bitch. The puppies of the bitch were allowed to walk freely in the facility.
 G: A drain 20 cm in width and 30 cm in depth.
 Unit: cm.

prepared from the suspension with PBS. The volume of inoculum per dog was 1 ml for all the routes, except the conjunctival route by which 0.1 ml was inoculated. The doses and routes employed are shown in Figs. 2 and 3.

Agglutination tests: Heat-killed *B. canis* antigen from strain QE-13 was kindly provided by Dr. A. Gohda of the Kitasato Institute. Serum agglutinin titers were determined by the microtiter method as previously reported [24].

Counting of *B. canis* cells from blood and urine: Blood samples were taken from the jugular or saphena vein. They were examined for bacteremia by incubating in TSB at 37°C for 7 days by the method described in the previous report [23]. The concentration of bacterial cells was estimated by counting *Brucella* colonies per 0.1 ml of blood culture on Trypticase soy agar (TSA, Eiken). Urine samples were collected weekly by the aid of a sterilized catheter from male beagles orally inoculated with *B. canis* (3.2×10^8) and placed under anesthesia with thiopental sodium (Ravonal, Tanabe). The concentration of *B. canis* cells in urine was estimated by counting *Brucella* colonies in TSA inoculated with 1 ml of diluted urine (1:10 to 1:10⁴ with sterilized PBS) [22].

Autopsy and detection of *B. canis* from the body: The dogs were sacrificed by exsanguination under anesthesia. The same organs and fluids as examined previously [22] were removed. The cut surface of each piece of the organs was stamped on TSA plate, while 0.1 ml of each fluid was spread on TSA plate. These plates were incubated at 37°C for 7 days. Identification of isolates was performed by the method described in the previous report [22].

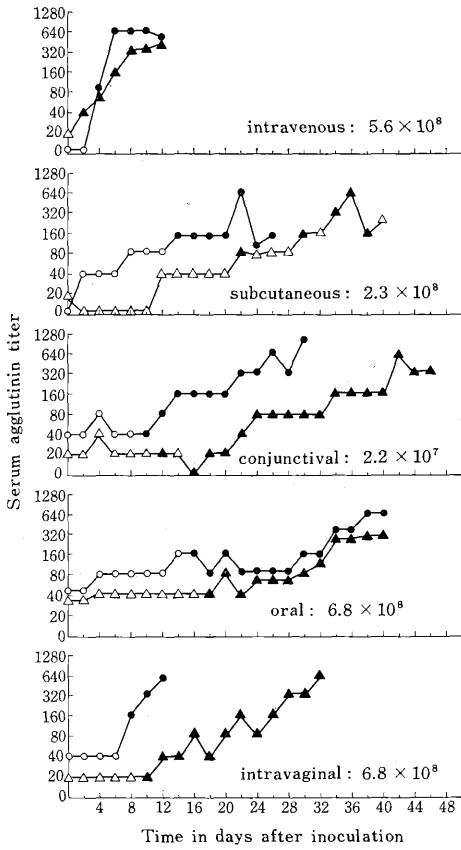
Maintenance of dogs: All the mongrels inocu-

lated with *B. canis* were kept individually in cages. The five male beagles and the bitch were chained in order to avoid contact with one another in an isolated facility (Fig. 1). Five puppies nursed by the bitch were allowed to walk freely within the facility. The concrete floor with a gentle slope was washed with water once a day. The dogs were fed a commercial diet of pellet and water in tubs.

Results

Inoculation with a large dose: All the adult mongrels inoculated with a large dose (approximately 10⁷ or 10⁸ organisms) were infected as exemplified by an increase in agglutinin titer (to 1:320 or higher) and by bacteremia (Fig. 2). Bacteremia started on the 2nd to 4th day by the intravenous route, on the 14th to 22nd day by the subcutaneous route, on the 10th to 12th day by the conjunctival route, on the 16th to 18th day by the oral route, and on the 8th to 10th day by the intravaginal route. Some of the inoculated dogs, especially those inoculated by the conjunctival and oral routes, were found to have bacteremia in spite of their low titers. In a dog inoculated by the subcutaneous route organisms were isolated intermittently from the blood after the onset of bacteremia. This dog showed anorexia and anaemia throughout the experiment.

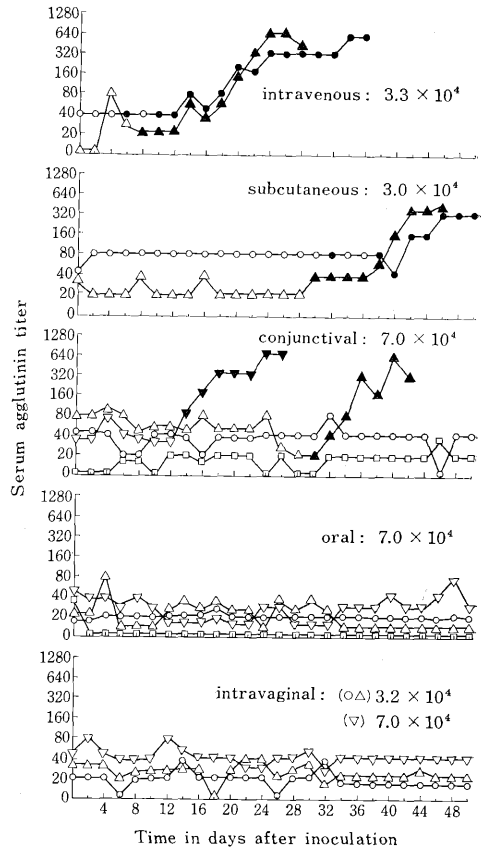
Fig. 2. Serum agglutinin titer and bacteremia after inoculation with approximately 10^8 (or 10^7) *B. canis* organisms



Remarks.
 ○ △ abacteremia; ● ▲ bacteremia.

Inoculation with a small dose: The results of inoculation with a small dose (approximately 10^4 organisms) are as follows (Fig. 3): 2/2 (No. infected/No. inoculated) (intravenous), 2/2 (subcutaneous), 2/4 (conjunctival), 0/4 (oral) and 0/3 (intravaginal). During the incubation period bacteremia and a significant level of agglutinin titer appeared later after inoculation with the small dose than that with the large dose by the intravenous and subcutaneous routes. In two of four dogs inoculated by the conjunctival route, *B. canis* was first isolated from blood samples collected on the 14th

Fig. 3. Serum agglutinin titer and bacteremia after inoculation with approximately 10^4 *B. canis* organisms



Remarks.
 ○ △ ▽ □ abacteremia; ● ▲ ▼ ■ bacteremia.

and 30th day, respectively, after inoculation. The other two dogs inoculated by the conjunctival route, four by the oral route, and three by the intravaginal route were abacteremic and showed low serum agglutinin titers throughout the observation period (for 50 days). At necropsy, no *B. canis* was isolated from any organs or fluid of the dogs examined.

Distribution of *B. canis* in the body: All the dogs confirmed to be infected were autopsied to examine the distribution of *B. canis* in organs and fluids when they demonstrated and agglutinin titer of 1:320 or

Table 1. Isolation of *Brucella* organisms from dogs inoculated with approximately 10^8 (or 10^7) *B. canis* organisms

Route	Intravenous		Subcutaneous		Conjunctival		Oral		Intravaginal			
Dose	5.6×10^8		2.3×10^8		2.2×10^7		6.8×10^8		6.8×10^8			
Days after inoculation	12	12	26	40	30	46	40	40	12	32		
Titer	480	480	160	240	800	320	640	320	640	640		
Blood	++	+	++	-	+	+	-	++	+	+		
Urine	-	-	+	-	ND	###	###	-	-	-		
Spinal fluid	-	-	-	-	ND	-	-	-	-	-		
	(L)	(R)	(L)	(R)	(L)	(R)	(L)	(R)	(L)	(R)	(L)	(R)
Heart	-	-	+	+	-	-	-	-	+	-		
Lung	++	++	+	+	-	-	-	++	-	+		
Liver	###	###	++	+	++	++	-	++	++	++		
Spleen	###	###	###	###	###	###	###	###	###	###		
Kidney Cortex	+	+	-	-	+	-	-	+	-	+	-	-
Medulla	+	++	-	-	+	-	-	+	-	+	+	+
Ureter	-	-	-	-	-	-	-	+	-	-	-	-
Urinary bladder	-	-	-	-	-	-	+	-	-	+	-	-
Bone marrow	++	C	+	-	+	+	-	-	++	++		
Lymph nodes												
Retropharyngeal	###	###	++	###	###	###	###	###	###	###	###	###
Mesenteric	++	++	++	++	++	++	++	++	++	++	++	++
Popliteal	###	###	++	-	###	###	++	++	###	###	++	++
Submandibular gland	+	-	-	-	-	-	+	-	+	-	-	-
Parotid gland	+	++	-	-	-	-	-	+	-	-	++	+
Prostate	+		-	++			###	###				
Epididymis	-	-	-	++	-	-	-	++	-	-	-	-
Testis	-	-	-	-	-	-	++	-	-	-	-	-
Uterus		-	-				-	-			-	-
Ovary		-	-				-	-			-	-

Remarks.

+, Less than 10 colonies per plate; ++, 11-100 colonies per plate; ###, More than 100 colonies per plate; ###, Confluent growth; -, Not isolated. ND: Not done. C: Contaminated.

higher with bacteremia. Results obtained are summarized in Tables 1 and 2. With regard to the fluids, *B. canis* organisms were isolated from 13 of 16 blood samples and from 6 urine samples; one of 6 infected females and 5 of 10 infected males. They were isolated from the spleen and lymph nodes at the highest rate and concentration of all the organs examined. These organs were followed by the liver and bone marrow. The organisms were isolated at low rate and concentration from the heart, lung, kidney, ureter, urinary bladder, and submandibular and parotid glands. A relatively large number of organisms were also isolated from

the following reproductive organs: prostate, epididymis, testis and uterus.

Excretion of *B. canis* in urine of orally infected males: Five male beagles orally inoculated with 3.2×10^8 *B. canis* organisms were examined for serum titer and the concentration of organisms in the urine and blood (Fig. 4). The organisms were detected from blood samples 2 to 6 weeks after inoculation. The concentration increased gradually to a level of 10^2 - 10^3 per ml of blood within 4 weeks after the first detection. This level was maintained to the end of an 18-week observation period. On the other hand, *B. canis* organisms were detected

Table 2. Isolation of *Brucella* organisms from dogs inoculated with approximately 10^4 *B. canis* organisms

Route Dose	Intravenous 3.3×10^4		Subcutaneous 3.0×10^4				Conjunctival 7.0×10^4					
	Days after inoculation		Titer		Titer		Titer		Titer			
	28	36	46	50	26	42						
	320	640	320	320	640	320						
Blood	+	++	—	++	++	+						
Urine	+	++	+	—	—	—						
Spinal fluid	—	—	—	ND	ND	ND						
	(L)	(R)	(L)	(R)	(L)	(R)	(L)	(R)	(L)	(R)		
Heart	+	—	—	—	—	—	—	—	—	—		
Lung	—	++	—	—	—	—	—	—	—	—		
Liver	++	++	—	++	—	++	++	++	—	—		
Spleen	+++	+++	+++	+++	+++	+++	+++	+++	+++	++		
Kidney Cortex	++	—	—	+	—	—	—	++	—	+	—	—
Medulla	++	—	—	+	—	—	—	—	—	+	—	—
Ureter	++	—	—	—	—	—	—	—	—	—	—	—
Urinary bladder	—	—	—	—	—	—	—	—	—	—	—	—
Bone marrow	++	—	—	—	—	—	—	—	—	—	—	—
Lymph nodes												
Retropharyngeal	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Mesenteric	++	—	++	—	—	—	++	—	++	—	—	—
Popliteal	+++	+++	+++	+++	++	++	+++	+++	+++	+++	+++	+++
Submandibular gland	—	—	—	—	—	—	—	—	—	—	—	—
Parotid gland	—	—	—	+	—	—	—	—	—	—	—	—
Prostate	—	—	++	—	+++	—	—	—	—	—	—	—
Epididymis	—	—	+++	+++	—	—	—	—	—	—	—	—
Testis	—	—	+++	+++	—	—	—	—	—	—	—	—
Uterus	—	—	—	—	—	—	—	—	++	++	—	—
Ovary	—	—	—	—	—	—	—	—	—	—	—	—

Remarks.

+, Less than 10 colonies per plate; ++, 11-100 colonies per plate; +++, More than 100 colonies per plate; +++, Confluent growth; —, Not isolated. ND: Not done.

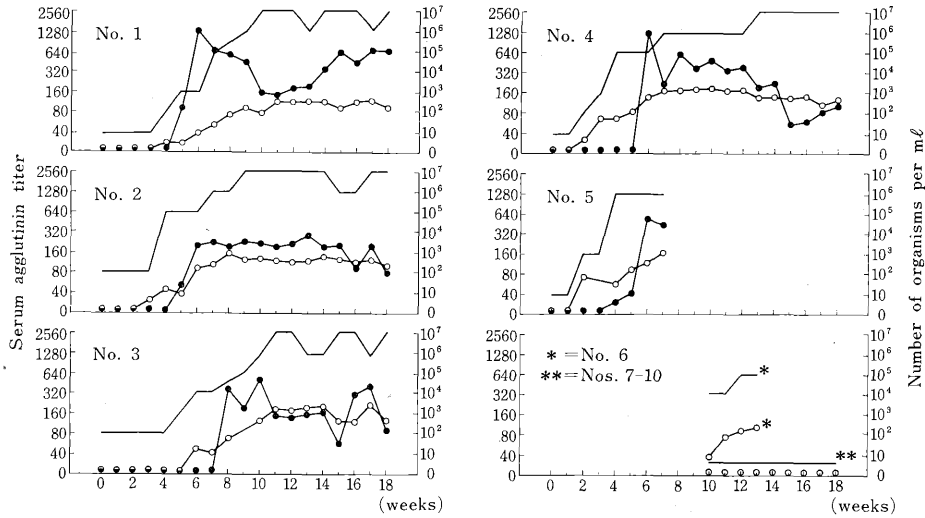
from the urine 1 to 4 weeks after the onset of bacteremia. Although they occasionally fluctuated in concentration in the urine, they were excreted continuously into the urine throughout the observation period. The highest concentration reached was 10^6 organisms per ml in 2 urine samples collected from 2 dogs. Serum agglutinin titers went up with the onset of bacteremia and were maintained at a high level thereafter.

Transmission in an isolated facility: Five intact 4-week-old puppies were housed together with the 5 infected male beagles since the time when the latter were inocu-

lated orally with *B. canis*. Blood samples were collected from the puppies in the 10th week of cohabitation. One of the puppies (No. 6, a 14-week-old female) showed bacteremia at the first sampling, when it had an agglutinin titer of 1:320 (Fig. 4). It was killed in the 13th week. *B. canis* was isolated from its organs and fluids as follows: +++, spleen and retropharyngeal and popliteal lymph nodes; ++, bone marrow; ++, liver; +, kidney, urinary bladder and urine. No organisms were detected from the heart, lung, submandibular or parotid gland, or uterus.

The other puppies remained abacteremic

Fig. 4. Transmission experiment with 5 males (Nos. 1-5) orally inoculated with *B. canis* (3.2×10^8) and 5 suppies (Nos. 6-10) born from a *B. canis*-free bitch



Remarks.

— Serum agglutinin titer; ○—○ *B. canis* in blood; ●—● *B. canis* in urine.

and developed no agglutinin during the 18-week observation period. No *B. canis* organisms were isolated from any puppy at necropsy made in the 18th week of cohabitation. The dam of every puppy was confirmed to be free from the infection by negative isolation of organisms from various organs and fluids at autopsy made in the 13th week of cohabitation of the puppies.

Discussion

Carmichael et al. reported that the transmission of canine brucellosis was accomplished by inoculation with approximately 10^9 organisms by various routes, including natural ones [4]. They estimated the dose necessary for inducing oral infection to be about 2×10^6 colony-forming units [3]. In the present experiment two of 4 dogs inoculated conjunctivally with approximately 10^4 organisms became infected, but dogs inoculated orally with the same dose did not. Therefore, it appears that the doses necessary for inducing conjunctival and oral

infection may be about 10^4 – 10^5 and 10^6 , respectively.

Although the concentration of viable *B. canis* organisms in the urine collected by bladder puncture has been reported by some workers [13, 21–23], no continuous observation has been made on the excretion of these organisms in the urine. The present experiment showed that *B. canis* organisms were excreted in the urine of males within a few weeks after the onset of bacteremia and that the excretion continued for at least 3 months. The highest concentration reached, that was as high as 10^6 per ml, appears to be sufficient for transmission.

Carmichael et al. reported that 2 uninfected bitches had been housed in an isolated unit together with 3 infected dogs (2 males and 1 female), but had not been infected within a 10-month observation period [2, 4]. They concluded that no transmission occurred readily with urine or feces. They did not describe, however, the concentration of organisms in the urine of

Table 3. Isolation of *Brucella* organisms from the urine and related organs of female dogs sacrificed at various times after experimental inoculation with *Brucella canis*

Days after onset of bacteremia	Route	Dose	Isolation of <i>Brucella canis</i>										
			Urine (No. of organisms/ml)	Urinary bladder	Kidney				Ureter		Uterus		
					Cortex L	Cortex R	Medulla L	Medulla R	L	R	L	R	
4	Intravaginal	10 ⁸	—	+	+	—	—	—	—	—	—	—	—
8	Intravenous	10 ⁸	—	—	—	—	—	—	—	—	—	—	—
12	Conjunctival	10 ⁴	—	—	—	—	—	—	—	—	‡	‡	‡
20	Intravenous	10 ⁴	+ (7.0 × 10)	—	‡	—	‡	—	‡	—	—	—	—
20	Conjunctival	10 ⁷	ND	—	+	—	—	+	—	—	—	—	—
22	Intravaginal	10 ⁸	—	—	+	—	‡	+	—	—	—	—	—

Remarks.

+, Less than 10 colonies per plate; ‡, 11–100 colonies per plate; ‡‡, More than 100 colonies per plate; ‡‡‡, Confluent growth; —, Not isolated. ND: Not done.

infected dogs.

In the present experiment, one (No. 6) of the 5 puppies which cohabited with infected males became infected. When it was examined for the serum agglutinin titer and bacterial concentration in the blood, bacteremia appeared to have occurred in the week of, or shortly before, the first examination. In the present experiment, the interval between the inoculation, except that by the intravenous route, and the onset of bacteremia varied from about 2 to 5 weeks. If this value is applicable to the puppy case, the transmission will be sure to have occurred several weeks before the onset of bacteremia. The most probable mode of transmission in this case may have been an intake of contaminated urine, since within these weeks *B. canis* organisms were excreted in the urine of 2 infected males (Nos. 1 and 4).

The concentration of 10⁶ organisms per ml seemed enough to transmit the infection, since the puppies were usually walking around the infected males in the facility. The reason why the puppies, but one, were not infected may be explained by the rare chance of contact with infective urine. If the puppies had been housed in a facility

small enough to provide frequent chances of direct contact with infective urine, all of them would have been infected.

Several other possible modes of transmission seem to be excluded. Although it is known that *B. canis* is excreted in semen [3], no venereal transmission must have occurred in the present experiment, because the puppies were immature. Transmission from the bitch, either congenital or via milk, must be denied, since the bitch was confirmed not to be infected. Transmission by saliva appears to be insignificant, because no organisms could be isolated from any salivary gland at a high concentration in the present experiment, as well as in the previous experiment [22]. As other sources of infection, feces and nostril fluids cannot be excluded completely, but they seem to be lower in probability than the urine. Munford et al. could not isolate *B. canis* organisms from ticks infesting bacteremic dogs [18]. The presence of a vector, however, was suggested from the present experiment in which it was impossible to eliminate mosquitoes completely from the facility used.

The distribution pattern of *B. canis* in the body was similar in all the infected dogs.

Table 4. Isolation of *Brucella* organisms from the urine and related organs of male dogs sacrificed at various times after experimental inoculation with *Brucella canis*

Days after onset of bacteremia	Route	Dose	Isolation of <i>Brucella canis</i>												
			Urine (No. of organisms/mL)	Urinary bladder	Kidney				Ureter		Prostate	Epididymis		Testis	
					Cortex L	Cortex R	Medulla L	Medulla R	L	R		L	R	L	R
10	Intravenous	10 ⁸	—	—	+	+	+	+	—	—	+	—	—	—	—
12	Conjunctival	10 ⁴	—	—	—	—	—	—	—	—	—	—	—	—	—
12	Subcutaneous	10 ⁸	+ (2.5 × 10 ²)	—	+	—	+	—	—	—	—	—	+	—	—
16	Subcutaneous	10 ⁴	+ (10)	—	—	—	—	—	—	—	+++	—	—	—	—
18	Subcutaneous	10 ⁴	—	—	—	+	—	—	—	—	—	—	—	—	—
18	Subcutaneous	10 ⁸	—	—	—	—	—	—	—	—	+	—	—	—	—
22	Oral	10 ⁸	—	+	—	—	—	—	—	—	—	—	—	—	—
24	Oral	10 ⁸	+++ (1.3 × 10 ⁵)	—	—	—	—	—	—	—	+++	—	—	—	—
30	Intravenous	10 ⁴	++ (8.0 × 10)	—	—	+	—	+	—	—	+++	+++	+++	+++	+++
34	Conjunctival	10 ⁷	+++ (ND)	+	—	+++	—	+++	—	+	+++	—	+	+++	—

Remarks.

+, Less than 10 colonies per plate; ++, 11-100 colonies per plate; +++, More than 100 colonies per plate; +++, Confluent growth; —, Not isolated. ND: Not done.

There was a certain correlation between the isolation of *B. canis* from the urine and that from male urogenital organs, regardless of the route or dose of inoculation. Data on the isolation of this organism from the urine and urogenital organs were pooled and analyzed by the time interval between the onset of bacteremia and the time of necropsy (Table 3 for females and Table 4 for males). The data seemed to suggest that *B. canis* organisms in urine might mostly be derived from such reproductive organs as the prostate, epididymis, and testis or the renal medulla rather than the bladder itself, at least in males.

There were several cases in which the onset of bacteremia was preceded by the elevation of serum agglutinin titer. It took about 30 days for some dogs infected by the natural routes to manifest the signs of infection. A quarantine period of more than 30 days may be recommended for canine brucellosis to check bacteremia and serum agglutinin titer repeatedly.

Although several natural human cases of canine brucellosis have been reported by

some workers [1, 3, 6-8, 10-12, 18, 25], no route of transmission of this disease from dog to man has been clarified as yet, even when an infected dog is present nearby the human patient. The present experiment may have shed some light on the possible source of infection in such human case.

Acknowledgments: The authors wish to thank Dr. A. Gohda, of the Kitasato Institute, for supply of antigen and also Dr. J. Yamada, of this Institute, for valuable advice throughout this investigation.

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要 約

イヌブルセラ症の伝播における尿の意義： 芹川忠夫・村口武彦（京都大学医学部附属動物実験施設）
—約 10^8 （又は 10^7 ）個の *Brucella canis* (*B. canis*) を種々の経路で接種した成犬は、全て感染が成立した。他方、約 10^4 個の菌を接種した結果は、次の通りであった。静脈内 (2/2), 皮下 (2/2), 眼結膜内 (2/4), 経口 (0/4) そして腔内 (0/3) (感染個体数/接種個体数)。さらに、*B. canis* で汚染した尿によるこの疾病の伝播を調べるために、5匹の雄犬は経口的に 3.2×10^8 個の *B. canis* を接種した時から、毎週尿中の *B. canis* の生菌濃度を調べ、そして *B. canis* フリーの母犬から生まれた5匹の4週令の仔犬と隔離した施設内で18週間、一緒に飼育した。5匹の仔犬のうち1匹に感染が成立した。*B. canis* は、菌血症の発現後、数週間以内に感染雄犬の尿中に排泄され、その後、常に高い濃度で分離された。それゆえ、この伝播は、*B. canis* で汚染した尿との接触によって起こったように思われた。