STUDIES ON BOVINE LEPTOSPIROSIS IN JAPAN

IV. BOVINE HEMOGLOBINURIA OCCURRING IN CATTLE IN THE NORTHERN PART OF HYOGO PREFECTURE, AND ISOLATION OF *LEPTOSPIRA AUSTRALIS* A, FROM CATTLE

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Bovine hemoglobinuria has long been found in the Japanese beef cattle raised in the northern part of Hyogo prefecture. The etiology of this disease was first studied in 1950 by WATANABE, et al.¹¹⁾, who isolated *Leptospira hebdomadis*, as a causal agent, from cattle which had succumbed to this disease. Their studies were followed by serological examinations on the sera of the recovered cattle by YAMAMOTO, et al.¹³⁾, the results of which indicated that some additional serotypes of leptospira could be etiologically participating in this disease. In 1953, in the same area, *Leptospira autumnalis* was isolated from the urine of cattle which showed hemoglobinuria by YANAGAWA, et al.¹⁴⁾. Thus, the bovine hemoglobinuria found in the northern part of Hyogo prefecture is considered to be caused by, at least, two or more serotypes of leptospira.

The authors have been engaged in the further investigation of this disease. In this paper, the first isolation of *Leptospira australis* A from cattle, will be described with some additional findings on this disease, which were the results of studies made in 1955.

MATERIALS AND METHODS

Isolation of leptospira: Urine of the affected cattle served as the material for the isolation of leptospira. Fifty ml of the urine was centrifuged for 30 minutes at 3,000 r.p.m., the upper part of the supernatant was then discarded, and the lower part was mixed with the sediment and was then inoculated, subcutaneously, into young guinea pigs weighing 250g. Body temperature of the guinea pigs was examined twice daily, and when fever paroxysm (more than 40°C) was observed in these guinea pigs, 2 ml of the blood was obtained by heart puncture. Each 1 ml of the blood was inoculated into each 10 ml of serum free KORTHOFF's medium which was then incubated at 25°C. Growth of leptospira in these mediums was microscopically examined on the 5th, 10th, 20th, and 30th days of incubation.

Serological methods: Sera of cattle were examined by agglutination-lysis test using the following 6 cultures as antigens; *L. icterohaemorrhagiae* (WEIL), *L. canicola* (H. Utrecht IV), *L. autumnalis* (Akiyami A), *L. hebdomadis* (Akiyami B), *L. australis* A (Akiyami C), and *L. pomona* (Suis S.R.), all of which were received from Dr. KITAOKA of the National Institute of Health. These cultures with *L. bataviae*, were also used for identification of newly isolated strains. For identification of newly isolated leptospira strains, each of the homologous immune rabbit serums was prepared, and the cross agglutination-lysis test, agglutinin absorption test (SCHÜFFNER-BOHLANDER³⁾), and PFEIFFER's test³⁾, were conducted. PFEIFFER's test was conducted by injecting, peritoneally, a mixture of 1 ml each of

Table 1. Result of Examinations on Affected Cattle from Which *Leptospira* Was Isolated

Cattle No.	Age	Onset of the signs of illness	Signs of illness	Agglutination-lysis test					Isolated leptospira		
				Ict.	Can.	Aut.	Heb.	Aust.	Pom.	Name of strain	Serotype
1	7	1955 Sept. 7.	Hemoglobinuria, anemia, jaundice, agalactia	3,000	100	300	—	10,000	100	Kimura	L. australis A
2	11	Sept. 18.	hemoglobinuria, anemia, jaundice, anorexia	1,000	1,000	3,000	—	1,000	100	Iwasaki	L. autumnalis
3	7	Sept. 19.	hemoglobinuria, anemia,	1,000	1,000	3,000	—	1,000	1,000	Nishimura	L. autumnalis
4	16	Oct. 5.	hemoglobinuria, anemia, jaundice	1,000	300	3,000	10	1,000	100	Kubota	L. autumnalis
5	4	Oct. 14.	hemoglobinuria, anemia, jaundice, diarrhea	3,000<	3,000<	3,000<	300	3,000<	3,000<	Horii	L. autumnalis
6	11	Oct. 17.	hemoglobinuria, anemia, jaundice	3,000	3,000<	3,000<	100	3,000<	1,000	Okabayashi	L. autumnalis

Note: 1) Numeral indicates the highest dilution of serum which was positive in agglutination-lysis test. — means negative at 1:10 serum dilution.

2) Ict.....L. icterohaemorrhagiae Can.....L. canicola Aut.....L. autumnalis

Heb.....L. Hebdomadis Aust.....L. australis A Pom ....L. Pomona

3) Cattle Nos. 1, 3, 4 and 5 were recovered, while No. 6 died, and No.2 was killed.

immune rabbit serum and *leptospira* culture into guinea pigs. Ascites of the guinea pigs were obtained at one-half, 2, and 4 hours, respectively, after the injection by means of the capillary pipette, and the lysis of *leptospira* were examined under the microscope.

RESULTS

In the northern part of Hyogo prefecture, one or two cattle are generally raised on each farm. In spring and autumn, it is usual for them to be pastured in the daytime in a common pasture of each block. In the autumn of 1955, hemoglobinuria was found in 30 of the total 7,063 cattle raised in the northern part of Hyogo prefecture. Jaundice, anemia and anorexia were shown beside hemoglobinuria, in these cattle. Examinations were undertaken in 18 cases. Sera of these cattle were examined by the agglutination-lysis test, the result of which indicated that 13 of them were infected with *L. autumnalis*, 2 with *L. hebdomadis*, 2 with *L. australis* A, and 1 with *L. icterohaemorrhagiae*. Symptoms were almost identical in these cattle, and no remarkable differences in the signs of illness was caused by the different serotypes of *leptospira*. Two of the animals died, and were shown, serologically to be infected with *L. autumnalis*, while the others recovered. Abortion was observed in one of the affected cows.

An attempt was made to isolate *leptospira* from the urine of these cattle with the consequent result that 6 strains were isolated from the urine of 6 of the cattle. The results of the examinations on these cattle are shown in Table 1. Attempts were also made to isolate *leptospira* from the aborted fetuses of 6 cows, one of them was that of an affected cow and the others were of serologically positive cows, which resulted in the negative.

Identification of these strains was conducted by the cross agglutination-lysis test, agglutinin absorption test, and PFEIFFER'S

Table 2. Cross Agglutination-lysis Test (1)

Antigen	Immune serum	Kimura
<i>L. icterohaemorrhagiae</i>		10
<i>L. canicola</i>		10
<i>L. autumnalis</i>		300
<i>L. hebdomadis</i>		—
<i>L. australis</i> A		10,000
<i>L. pomona</i>		10
<i>L. bataviae</i>		—
Kimura		30,000

Numerals indicate the highest dilution of serum which was positive in agglutination-lysis test.— means negative at 1:10 serum dilution. These foot-notes are also applicable in Tables 3–5.

Table 3. Cross Agglutination-lysis Test (2)

Immune serum	Antigen	Agglutination-lysis titer
<i>L. icterohaemorrhagiae</i>	<i>L. ictero.</i> Kimura	1,000 —
<i>L. canicola</i>	<i>L. canicola</i> Kimura	3,000 —
<i>L. autumnalis</i>	<i>L. autumnalis</i> Kimura	3,000 10
<i>L. hebdomadis</i>	<i>L. hebdomadis</i> Kimura	3,000 —
<i>L. australis</i> A	<i>L. austr. A</i> Kimura	30,000 30,000
<i>L. pomona</i>	<i>L. pomona</i> Kimura	3,000 —
<i>L. bataviae</i>	<i>L. bataviae</i> Kimura	3,000 —

Table 4. Agglutinin Absorption Test

Antigen	Serum	Kimura		
		Original	Absorbed with	
			Kimura	<i>L. australis</i> A
Kimura		6,400	—	—
<i>L. australis</i> A		3,200	—	—

Our strain is the third in Japan. However, no report on the isolation of *L. australis* A from cattle has been available up to the present, this being the first report on *L. australis* of bovine origin.

From the results of investigations on the bovine hemoglobinuria carried out in the northern part of Hyogo prefecture by WATANABE, et al., YANAGAWA, et al., and by the authors of the present paper, it is obvious that three types of leptospira; *L. hebdomadis*, *L. autumnalis* and *L. australis* A, are the causal agents of this disease. With regard to the frequency of these serotypes, it is known, from the results of the typing of the isolated strains, and the serological survey, that *L. autumnalis* occurs most frequently, *L. hebdomadis*

test. From the results of these tests it is indicated that 5 strains (Iwasaki, Nishimura, Kubota, Horii, and Okabayashi) belong to *L. autumnalis*, while another (Kimura) is, as indicated in Tables 2–5, antigenically corresponded to the *L. australis* A. Inoculation of these newly isolated strains into guinea pigs resulted in febrile response but not in the death of these animal.

A cattle from which the Kimura strain was isolated, was raised on a farm of the Kuwada block, Wadayama town, Hyogo prefecture, and presented hemoglobinuria on September 7, 1955. One month later, on October 11, sera of all the 41 cattle of this block were examined by the agglutination-lysis test. The result of the test indicated that 8 (19%) of them were positive at a 1:1,000 or higher serum dilution, without any signs of illness, and that these were considered to be infected with any of the following; *L. autumnalis*, *L. hebdomadis* or *L. australis* A.

The monthly occurrence of bovine hemoglobinuria was observed in this area from 1953 to 1955. A total of 45 cases were found within this period of time, most of which occurred in the months of September and October, as indicated in Table 6. Number of the cases by age is shown in Table 7. A small number of the cases were found among calves, while most of the cases involved cattle of more than three years of age, and the morbidity was found to grow higher with age.

DISCUSSION

In Japan, *L. australis* A has been isolated only twice, by SHIOZAWA and KUBO⁶⁾, in 1937, and by TSUBOSAKI and AOKI¹⁰⁾, in 1954, both of these strains are of human origin.

follows next, and *L. australis* A is to be found but seldom. These findings in cattle are

Table 5. Pfeiffer's Test†

Immune serum	Leptospira	Result
<i>L. autumnalis</i>	Kimura	—
<i>L. hebdomadis</i>	"	—
<i>L. australis</i> A	"	+
Kimura	"	+

+ indicates lysis, — no lysis.

Reading of the result was made at one-half, 2 and 4 hours after injection, and the same result was obtained.

Table 6. Monthly Occurrence of Bovine Hemoglobinuria

August			September			October			November		
B.	M.	L.	B.	M.	L.	B.	M.	L.	B.	M.	L.
4	0	1	8	8	5	5	7	2	3	0	0
B....Beginning			M....Middle			L....Last					

Table 7. Morbidity of Bovine Hemoglobinuria by Age

Age	Number of		Morbidity %
	Cattle	Cases	
1	3,134	2	0.06
2	286	0	0
3	285	1	0.35
4	435	4	0.92
5	452	5	1.11
6	470	6	1.28
7	506	5	0.99
8	399	4	1.00
9	356	2	0.56
10	214	0	0
11	185	4	2.16
12	171	4	2.33
13	114	5	4.39
14	31	2	6.45
15	18	0	0
≥16	7	1	14.29
Total	7,063	45	0.64

L. australis A was isolated from a cattle which was affected with hemoglobinuria, in 1955, in the northern part of Hyogo prefecture. This strain is considered to be the first *L. australis* A of bovine origin. Five strains of *L. autumnalis* were also isolated from each of the cattle which showed hemoglobinuria. Thus, bovine hemoglobinuria occurring in the northern part of Hyogo prefecture was proved to be attributed to at least three types of leptospira; *L. autumnalis*, *L. hebdomadis*, and *L. australis* A. No remarkable difference in the signs of illness was caused by these different serotypes. This disease is found from August to November, particularly in September and October. A few incidence of this disease was found in calves, and the morbidity grows higher with age.

similar to those in the case of human leptospirosis occurring in Japan, which were described by KITAOKA⁷⁾, with the exception that no case of isolation of *L. icterohaemorrhagiae* from cattle has been reported.

Bovine leptospirosis in this area has been found to occur from August to November. Recently, however, the authors were able to isolate *L. autumnalis* from cattle, in April. This case, which could be interesting from the epizootiological viewpoint, will be described in the following paper of this series.

SIPPEL, et al.⁹⁾ described that, in America, bovine hemoglobinuria occurs more frequently in young cattle than in adult cattle, the results of the present study show, however, that most of the cases were found in cattle which were older than three years of age.

Abortion has been found, in this area to occur a little later to the infection, and a mummified or a semi-mummified fetus was frequently delivered. From the fact that the abortion occurs in those cows affected with hemoglobinuria and in latently infected cows, and that the sera of these aborted cows are known to be positive in agglutination-lysis test but negative in the tests of infectious abortion in cattle such as brucellosis, vibriosis and trichomoniasis, possible relationship has been considered between these abortions and leptospirosis, as in some other countries^{1,2,4,5,8,9,12,16)}. Attempts have been made to isolate leptospira from these fetuses but the results were negative. The relationship is, therefore, still worthy of further investigation.

SUMMARY

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牛レプトスピラ病に関する研究

IV. 兵庫県北部地方における牛の血色素尿症ならびに
Leptospira australis A の分離について

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1955年秋、兵庫県北部（但馬地方）に飼育されている 7,063 頭の和牛のうち 30 頭に血色素尿症がみとめられたので、これらの病牛についてレプトスピラ病の調査を行なった。また 1953—55 年に、同地方で、本病の発生時期および年令別罹病率を観察した。調査の結果を要約すると、つぎのとおりである。

1. 血色素尿症を示した牛のうち 18 頭の血清について、凝集溶菌反応を行なったところ、13 頭が *L. autumnalis* に、2 頭が *L. hebdomadis* に、2 頭が *L. australis* A、および 1 頭が *L. icterohaemorrhagiae* に陽性を示した。*L. autumnalis* に陽性を示した牛 2 例が斃死したが、その他はいずれも回復した。回復例では、レプトスピラの型によって症状に軽重の差がみられることはなかった。

2. 上記の 18 頭の発病牛の尿についてレプトスピラ分離を試みた結果、6 株のレプトスピラが分離された。

3. この 6 株のレプトスピラを、交叉凝集溶菌反応、凝集素吸収試験および PFEIFFER 反応によって、同定したところ、このうち 5 株は *L. autumnalis*,

他の 1 株(木村株)は *L. australis* A と同定された。牛からこの型のレプトスピラが分離されたのは、はじめてと考えられる。兵庫県北部地方の牛血色素尿症の原因として、すでに分離されている *L. autumnalis* および *L. hebdomadis* とあわせて、結局 3 つの型のレプトスピラが現在までに確認されたことになる。

4. 木村株が発病牛から分離された兵庫県朝来郡和田山町久田和部落で、この牛の発病後約 1 カ月目に、41 頭の全飼育牛の血清を得て凝集溶菌反応を行なったところ、凝集溶菌価 1:1,000 またはそれ以上を示した牛が 8 頭(19%) みとめられた。これらは *L. autumnalis*, *L. hebdomadis* および *L. australis* A のいずれかひとつに陽性反応を示した。しかし、発病した牛は木村株が分離された牛だけで、その他の牛では症状をみとめていない。

5. 1953—55年に兵庫県北部地方でみとめられた牛の血色素尿症は 45 例で、その大部分が 9 月または 10 月に発病した。そして発病は 3 才以上の成牛に多く、年令とともに発病率が高くなる傾向がみとめられた。