STUDIES ON *HEPATITIS CONTAGIOSA CANIS* I.

INFECTION EXPERIMENTS ON DOGS WITH TWO STRAINS OF THE VIRUS AND SEROLOGICAL INVESTIGATIONS WITH THE COMPLEMENT-FIXATION TEST

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INTRODUCTION

Since RUBARTH (1947) first reported a new viral disease of dogs, *Hepatitis contagiosa canis* (H. c. c.) in Sweden, incidences of the disease have been reported one after another from many other countries. In Japan, the incidence of this disease was demonstrated in 1953 for the first time by FUJIMOTO et al. of the Department of Veterinary Pathology of this University. They observed characteristic lesions in the 4 puppies of a 6-months-old litter. Since then, they report that their autopsied cases dead from this disease have reached more than 10 cases including foxes up to the present. In 1954, the authors had the opportunity to attempt some inoculation experiments on puppies with liver suspensions of 2 naturally infected dogs in Sapporo.

The results of this experiment were reported at the 39th Meeting of Jap. Soc. Vet. Sci., in April, 1955. OCHI et al. also made transmission experiments with the materials from a naturally infected 10-months-old Spitz in Tokyo; they compared the disease set up by known H. c. c. virus strains sent from U. S. A. and their own. Their studies were presented at the 3rd Meeting of Jap. Soc. Virology, in April, 1955 at the almost same time when the present authors' work was reported.

The present article describes serial transmission experiments on puppies with strains of H. c. c. virus found in Sapporo and clinical features of those infected animals. Furthermore, the rise and fall of complement-fixing antibody of the experimental cases and the distribution of C. F. antibody among dogs in several districts of Japan were investigated.

TRANSMISSION EXPERIMENT ON PUPPIES

Materials and Methods: H. c. c. virus strains: "YAMAGUCHI" strain, from a 6-months-old male dog (Tosainu, a Japanese breed of dog) autopsied in January 20, 1954, the other, "MATSUDA" strain, from a 5-months-old male Shepherd autopsied on February 8, 1954. An elevation of body temperature and the loss of appetite were marked in these 2 cases. Macroscopically, edema of the gall bladder wall and tonsillitis connected with other autopsy findings suggested H. c. c. Touch preparations from the livers of these cases indicated the presence of the nuclear inclusions to be specific of H. c. c. These 2 cases also histopathologically diagnosed as H. c. c. by the Department of Veterinary Pathology.

In the experimental infections, the authors used chiefly 10% saline suspension of the liver. Information on injection routes, doses and other necessary items are listed in table 1.

The dogs employed for these experiments were all mongrel puppies of 14 to 90 days old including 18 puppies from 7 litters as is also indicated in table 1.

Results: Fig. 1 indicates the results of the serial passage of liver suspensions on puppies. Four consecutive passages were positively made with the "MATSUDA" strain. At the 1st to 3rd passage one or several puppies died suffering from the disease, however at the 4th passage, no fatal infection occurred, although mild and chronic infections could be recognized in all of eight survivors. Establishment of infection was decided by fever, leucocyte count and other general signs of illness and pathological findings characteristic to this disease, also by complement-fixation test. In the fatal cases, inclusion bodies typical of infectious hepatitis were always found. On the other hand, in the dogs infected with the "YAMAGUCHI" strain, none of ten puppies either developed severe clinical symptoms or died.

In case of the "MATSUDA" strain, death occurred in 8 out of 28 (28.6%) in comparison with 0% in the "YAMAGUCHI" strain. Mild infection also occurred

FIG. 1. *Schema of Experimental Transmission to Puppies*

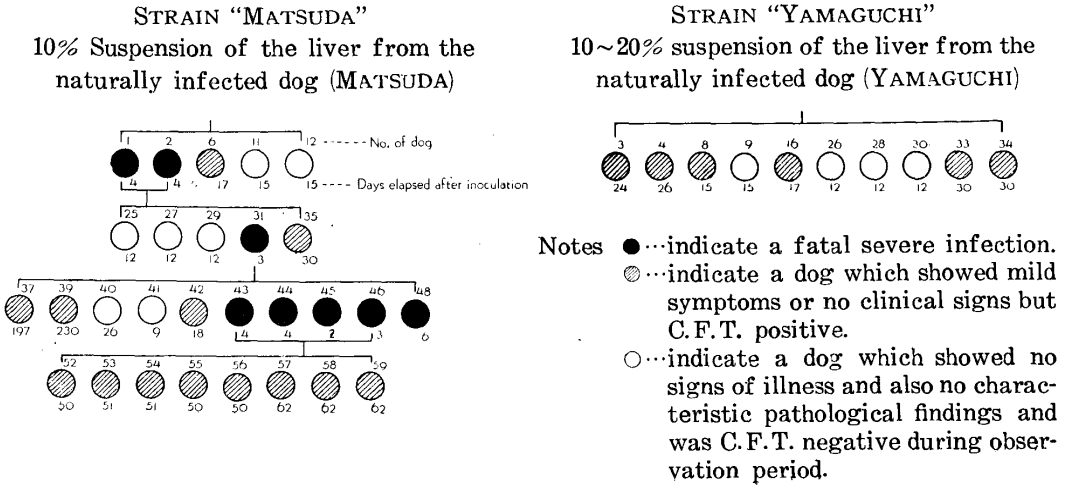


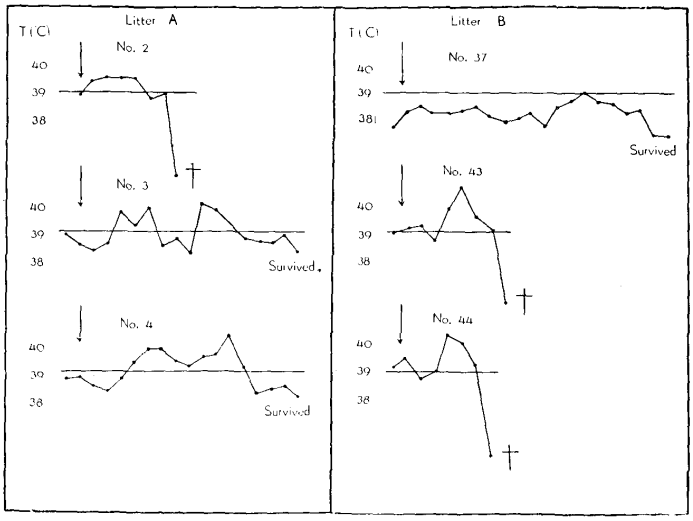
TABLE 1. *Description of Dogs Used for Experimental Infections*

STRAIN OF VIRUS	PASSAGE	NO. OF DOG	DAYS AFTER BIRTH	MATERIAL FOR INOCULATION	ROUTE	DOSE (ml)	TERMINATION (in days)		COMPLEMENT-FIXATION TITRES	PRESENCE OF INCLUSION BODY	DESIGNATION OF THE LITTER	REMARKS
MATSUDA	1	1	45	20 % suspension in saline of the liver of a field case "MATSUDA"	i. p.	15	4	Death		+	·	
		2	90		i. p., s. c.	20, 5	4	"		+	A	
		6	45		i. p., s. c.	20, 5	17	Killed	1 : 16	—	·	
		11	14		s. c., i. p.	3, 9	15	"		—	·	
		12	"		i. p.	"	"	"		—	·	
	2	25	25	10 % suspension in saline of the liver of dog Nos. 1 and 2	i. v.	3	12	"	——	—	B	
		27	"		i. p.	5	"	"	——	—	B	
		29	20		i. p., i. v.	15, 1.5	"	"	——	—	C	
		31	·		i. v.	3	3	"	——	+	·	
		35	30		"	"	30	"	1 : 64	—	D	Corneal opacity
	3	37	48	10 % suspension in saline of the liver of dog No. 31	i. v.	3	197	"	1 : 32	—	E	"
		39	·		"	"	230	"	"	—	·	"
		40	40		"	"	26	"	——	—	·	
		41	·		"	"	9	"	——	—	·	
		42	·		"	"	18	Death	1 : 32	—	·	
		43	48		"	"	4	"		+	E	
		44	48		"	"	"	"		+	E	
		45	41		"	"	2	"		+	·	
		46	45		"	"	3	"		+	·	
		48	30		"	"	6	Killed		+	·	
	4	52	31	10 % suspension in saline of the liver of dogs Nos. 43, 44, 45 and 46	i. v.	2	50	"	1 : 16	—	F	
		53	21		"	3	51	"	1 : 8	—	G	
		54	30		"	"	"	"	"	—	G	
		55	"		"	"	50	"	1 : 16	—	F	
		56	"		"	"	"	"	1 : 8	—	·	
		57	"		"	"	62	"	"	—	·	
		58	"		"	"	"	"	1 : 32	—	·	
		59	"		"	"	"	"	1 : 8	—	·	
YAMAGUCHI	1	3	90	20 % suspension in saline of the liver of a field case "YAMAGUCHI"	i. p., s. c.	20, 5	24	"	1 : 32	—	A	
		4	"		i. p.	20	26	"	1 : 8	—	A	
		8	14		s. c.	3	15	"	1 : 16	—	·	
		9	"		i.p., Oral	9, 3	"	"	——	—	·	
		16	45	10 % suspension in saline of the liver of the same dog	i. p.	15	17	"	1 : 16	—	·	
		26	25		i. v.	3	12	"	——	—	B	
		28	"		i. p.	5	"	"	——	—	·	
		30	20		i. p., i. v.	2.5, 1.5	"	"	—	—	C	
		33	30		i. v.	2.5	30	"	1 : 32	—	D	Corneal opacity
		34	"		"	"	"	"	"	—	D	

in 13 cases out of 28 (46.4%). Fatal, severe and mild or inapparent infection were recognized in a total of 21 out of 28 (75%) in case of the "MATSUDA" strain, while in case of the "YAMAGUCHI" strain, only mild infections occurred in 6 out of 10 (60%).

Susceptibility of puppies to H. c. c. virus even in the same litter seems to be widely variable. This can be seen from chart 1 and table 1. Therefore, as for the present differences of the severity of clinical signs and mortality rates between these 2 virus strains, the authors would have no explanation.

CHART 1. *Course of Disease Observed in Puppies from 2 Litters*



Notes: No. 2 ··· 20% suspension of the liver of a naturally infected dog (the strain "MATSUDA") i. p. 20, s. c. 5 ml.
No. 3 ··· 20% suspension of the liver of a naturally infected dog (the strain "YAMAGUCHI") i. p. 20, s. c. 5 ml.
No. 4 ··· 20% suspension of the liver of a naturally infected dog (the strain "YAMAGUCHI") i. p. 20 ml.
Nos. 37, 43 and 44 ··· 10% suspension of the liver from dog No. 31-2nd generation of the starin "MATSUDA", i. v. 3 ml, respectively.

CLINICAL SIGNS OF ILLNESS OBSERVED IN THE EXPERIMENTAL CASES

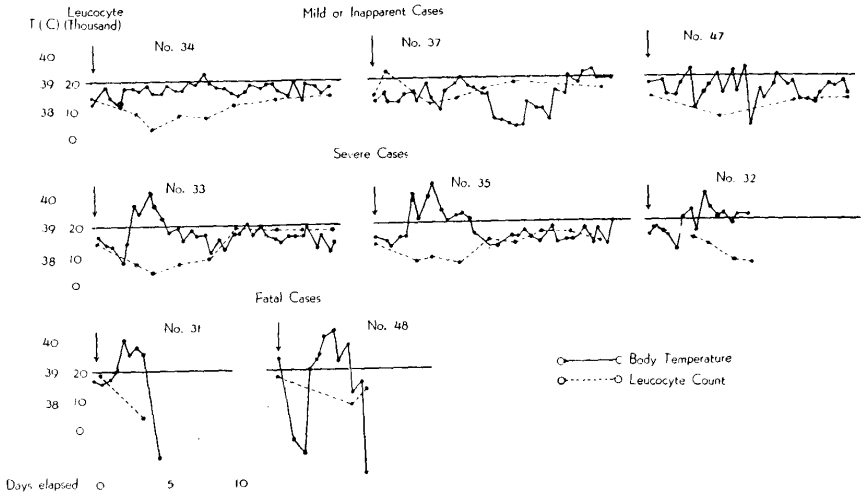
After inoculation, temperatures were taken twice each day and a leucocyte count was made daily. Other general signs of illness were observed and are shown in table 2. There are many reports on clinical and hematological findings by MCSHERRY, GILLESPIE, COFFIN, SCHILLING, PARRY, SMITH, VERGE, etc. In the authors' cases, the clinical pictures and their severity were varied case by case. Sudden febrile attack appeared within 1~2 days in almost all fatal cases followed by complete loss of appetite. Marked decrease of leucocytes (ranging from 3,500 to 8,100) was seen in the febrile stadium in mamy cases

but not in all. The grade of leucopenia varied with the individual case. The other clinical signs such as tonsillitis, diarrhea, conjunctivitis, vomiting, swelling of submaxillary lymph node and prolonged coagulation of the blood etc. were accompanying symptoms often as many workers have already reported. Severity of the clinical course seems not to be dependent upon the dose or route of injections. Corneal opacity was seen in 4 cases out of 10 severely infected cases. This corneal cloudiness appeared 6~19 days, chiefly 6~8 days after intravenous inoculation when all the other clinical signs had disappeared. Jaundice, gingival hemorrhage, subcutaneous edema on the head or neck and mental excitement could not be recognized in the authors' cases. Concerning fever reaction and deviation of leucocyte count in some of the typical form of fatal, severe and mild or inapparent cases data are graphed in chart 2.

TABLE 2. *Clinical Symptoms Observed in Experimentally Infected Dogs*

CLINICAL SIGNS	FATAL CASES (No. of Dog)								NON-FATAL CASES (No. of Dog)									
	1	2	31	43	44	45	46	48	3	4	6	16	32	33	34	35	37	39
Fever attack	○	○	○	○	○	○	○	○	○	○	○	○	○	○	•	○	•	○
Partial or complete loss of appetite	○	○	○	○	○	○	○	○	○	○	○	○	○	○	•	○	○	•
Tonsillitis	○	○	•	○	○	•	○	•	•	•	•	•	•	•	•	•	○	•
Conjunctivitis	•	•	•	•	•	•	•	○	•	•	•	•	•	•	•	•	•	•
Keratitis	•	•	•	•	•	•	•	•	•	•	•	•	•	○	•	○	○	○
Diarrhea	○	○	○	•	•	○	•	○	○	•	○	•	•	○	○	•	•	•
Vomiting	○	○	•	•	•	•	•	•	•	•	○	•	•	•	•	•	•	•
Swelling of the submaxillary lymph node	○	○	•	○	○	○	○	•	○	○	○	•	•	•	○	•	○	•
Leucopenia	○	○	○	?	?	?	○	○	•	•	○	•	•	○	○	○	○	○
Prolonged coagulation of the blood	•	•	○	•	•	•	•	○	•	•	•	•	•	•	•	○	○	•

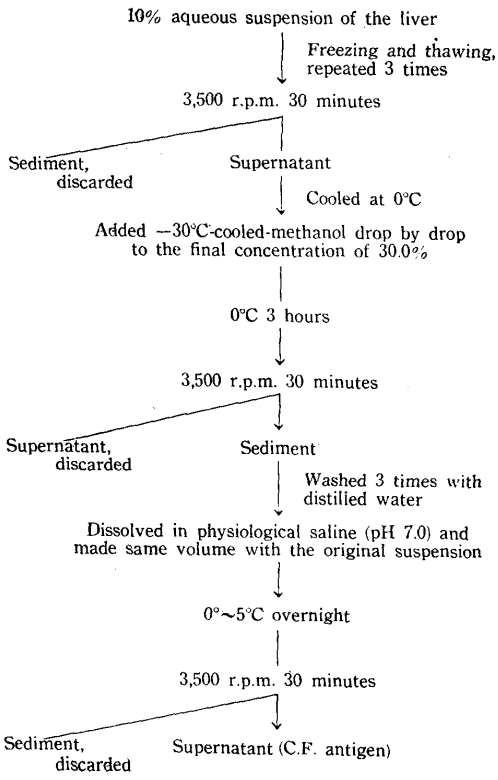
CHART 2. *Clinical Course and Leucocyte Count*



COMPLEMENT-FIXATION TEST

Preparation of Antigen: Several kinds of complement-fixing antigens were prepared from the liver of naturally or experimentally infected fatal cases following LARIN[7]. Negative antigens were also prepared by using young healthy puppy liver. The method used for the preparation of antigens were methanol- and ethanol-precipitation and also absorption and elution with kaolin, alumina and diatom. All of these preparations indicated almost the same antigenicity as a complement-fixing antigen. In these experiments, the authors used the methanol precipitation method following LARIN[7] as indicated in Fig. 2.

FIG. 2. *Preparation of Methanol-Precipitated Antigen*



Technique of Test: Several preliminary tests were performed as to sensitizing hours, temperature, quantitative relationship between antigen and antibody etc.

One of the most important points seems to be incubation time for sensitization. From the results indicated in tables 3 and 4, the time sufficient and necessary for sensitization seem to be 2 hours at 37°C. Overnight sensitization at low temperature sometimes results in non-specific reactions.

The optimum conditions for the test were understood by the authors as follows:—

1. Hemolytic system contained equal part of 3% sheep blood and 3 units of sheep hemolysin.
2. Incubation temperature was 37°C in water bath.
3. Incubation time for preliminary test, such as titration of hemolysin, complement and anticomplementary activity of antigen, was 30 minutes.
4. Tests for complement-fixing antibody were made using mixtures consisting of 0.25 ml amounts of serial twofold dilutions of inactivated serum (30 minutes at 56°C), 0.25 ml of the appropriate dilutions of antigen (usually 2 or 4 units) and 0.5 ml of guinea pig complement containing 2 full units. Each test tube of above mixtures was given primary incubation in water bath at 37°C for 2 hours, following which time 0.5 ml of sensitized sheep red cells was added and the test was read after a second incubation at 37°C for 30 minutes. Titers are given as the highest dilution of serum showing 2 or more fixation of complement.

TABLE 3. *Experiments on Sensitizing Hours, at 37°C*

SENSITIZATION		SERUM NO.																				
Temperature	Hours	4							5						16							
		×2	×4	×8	×16	×32	×64	×2*	×8	×16	×32	×64	×128	×8*	×2	×4	×8	×16	×32	×64	×2*
37 °C	30 min.	1	0	0	0	0	0	0	2	1	±~0	0	0	0	0	0	0	0	0	0	0
"	60 "	4	3	1	1~±	0	0	0	4	3	2	1	0	0	1	2	1	±	0	0	0
"	120 "	4	4	3	1	1	0	0	4	4	4	3	1	0	4	4	4	1	0	0	0

Notes: 1. Figures 4, 3, 2 and 1 indicate 100, 75, 50 and 25% fixation of complement, respectively.
2. 0 means complete hemolysis, ± incomplete hemolysis less than 1.
* Serum control

TABLE 4. *Experiments on Sensitizing Temperature*

SENSITIZATION		37 °C 1 Hour											0~5°C Overnight									
ANTIGEN		Positive					Negative						Positive					Negative				
		×4	×8	×16	×32	×64	×4	×8	×16	×32	×64	×4*	×4	×8	×16	×32	×64	×4	×8	×16	×32	×64	×4*
SERUM NO.	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	4	3	3	0
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	4	4	3	0
	4	1	0	0	0	0	0	0	0	0	0	0	4	4	4	1	0	2	3	3	3	3	0
	5	4	4	4	3	1	0	0	0	0	0	0	4	4	4	4	3	4	4	4	4	3	0
	13	0	0	0	0	0	0	0	0	0	0	0	4	2	0	0	0	1	1	2	1	1	0
	33	0	0	0	0	0	0	0	0	0	0	0	4	1	0	0	0	1	1	1	1	1	0
	34	2	1	0	0	0	0	0	0	0	0	0	4	4	4	1	0	2	2	2	2	1	0
	35	2	1	1	0	0	0	0	0	0	0	0	4	4	4	4	0	2	2	2	2	2	0

* Serum control

TABLE 5. *Titration of Methanol-Precipitated Antigen*

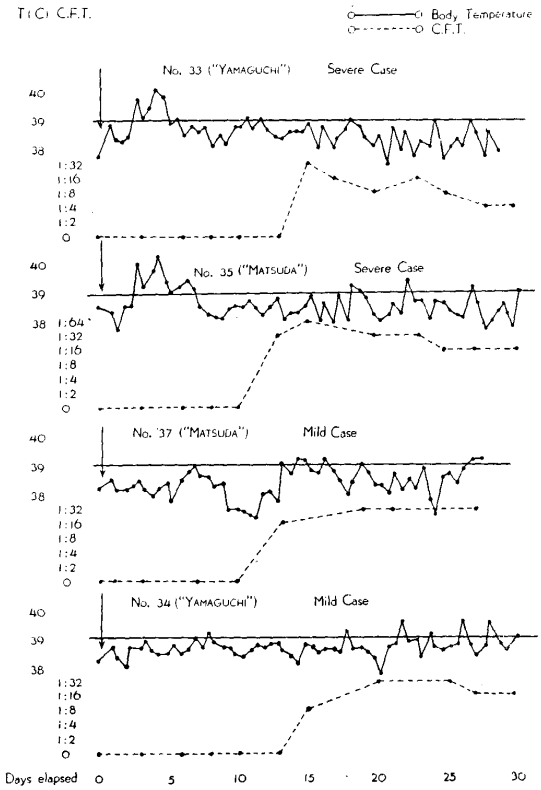
ANTIGEN		SERUM NO. 34 ×4	×8	×16	×32	×64	×128	×256	×4*	SERUM NO. 35 ×4	×8	×16	×32	×64	×128	×256	×4*	Negative serum ×4	×8	×16	×32	×64	×4*
1	× 0	4	4	4	4	0	0	0	0	4	4	4	4	1	0	0	0	1	1	0	0	0	0
	× 2	4	4	4	1	±	0	0		4	4	4	4	±	0	0		0	0	0	0	0	
	× 4	4	4	4	4	0	0	0		4	4	4	4	0	0	0		±	0	0	0	0	
	× 8	4	4	4	1	0	0	0		4	4	4	4	±	0	0		0	0	0	0	0	
	× 16	4	4	2	1	0	0	0		4	4	4	1	0	0	0							
	× 32	2	1	1	±	0	0	0		4	1	2	±	0	0	0							
2	× 0	4	4	4	3	±	0	0		4	4	4	4	1	0	0		±	0	0	0	0	
	× 2	4	4	4	2	±	0	0		4	4	4	4	1	0	0		0	0	0	0	0	
	× 4	4	4	4	2	0	0	0		4	4	4	4	±	0	0		0	0	0	0	0	
	× 8	4	4	4	1	0	0	0		4	4	4	3	±	0	0							
	× 16	4	±	0	0	0	0	0		4	4	1	±	0	0	0							
	× 32	2	0	0	0	0	0	0		1	±	0	0	0	0	0							

* Serum control

Appearance and Persistence of C. F. Antibody in Experimental Dogs

In the authors' experimental cases, C. F. antibodies appeared during 12~15 days after inoculation. Several cases are indicated in chart 3. The severity of the clinical manifestations of the infection and the rise of C. F. antibody seem to have no correlation. Even in a case which shows no detectable clinical signs, C. F. antibody could be detected within the days above-noted. Usually it seems to reach the maximum titre in 15~20 days after inoculation and persists for considerably long period. In one case of the authors, almost the same high titre of C. F. antibody as in its initial stage was demonstrated throughout the course of 7 months. On the other hand, inclusion bodies in the liver were not detected in any case of the heavily infected, C.F.T. positive dogs sacrificed at 17~30 days after inoculation.

Chart 3. *Complement-Fixation Test on Experimental Cases*



Distribution of C. F. Antibodies in the Dog from Several Districts of Japan

With regard to the distribution of C. F. antibodies among dogs, studies have not yet been made in this country. The authors undertook to carry out a survey concerning it and collected several hundred blood samples from Sapporo, Tokyo, Hiroshima and Gifu. Complement-fixation tests were carried out as soon as possible after receipt of dog sera. The serum sample was usually inactivated at 56°C for 30 minutes, however the serum which shows anticomplementary activity, was additionally inactivated at 60°~65°C for 30 minutes. The serum samples which showed non-specific reaction with the negative antigens were of course omitted from this survey. The specific reactions showing more than 50% fixation of complement in serum starting from serial twofold dilutions were considered as positive.

This experiment was carried out in 1954. The results showed the high positive rate of C. F. antibody ranging from 44 to 57% in each district. The data seem to indicate that considerably heavy contaminations of H. c. c. virus have occurred

already in this country as was recognized in several other countries by RUBARTH in Sweden, BRUNNER et al. in Switzerland, SCHEU in Germany, FLORENT et al. in Belgium and LEHNERT in Sweden.

TABLE 6. *Distribution of Complement-Fixing Antibody in the Dog Sera from Several Districts of Japan*

DISTRICT	NO. EXAMINED	NO. POSITIVE	(%)
Sapporo	172	77	(44)
Tokyo	100	57	(57)
Hiroshima	50	27	(54)
Gifu	50	25	(50)
Total	374	186	(49.7)

CONSIDERATION

In the present transmission experiments, comparatively high rates of infections were observed in both series although many of these clinical course were mild. With the "MATSUDA" strain, fatal infection could be transmitted to the 3rd passage, however it did not occurred in the 4th passage. On the other hand, with the "YAMAGUCHI" strain, none of the infected puppies suffered death. The dogs used in this experiment were all healthy young puppies aged from 2 weeks to 3 months without any sign of the presence of C. F. antibody before use. However about 26.3% (11 cases out of 38) of them were not infected by inoculation of heavy dose, perhaps several hundred times the minimum infection dose employed by STÜNZI and POPPENSIEK or by LARIN[8]. Even in dogs from the same litter, the clinical manifestations of infections widely deviated in spite of the use of intravenous inoculations of the same dose. As to the susceptibility of puppies to this virus, there seem to remain many problems, as many authors e. g., RUBARTH, GORET et al. SCHILLING etc. have already stated. Accordingly, as for the reason of the differences of the results obtained from transmission experiments between the two strains "YAMAGUCHI" and "MATSUDA" the authors have no explanation.

Concerning the appearance of C.F. antibody, LARIN (1951, 1953) reported 21～25 days after inoculation, 12～38 days after clinical signs[7] or 12 days to 6～7[8] weeks, VERGE and PARAF at the 7th day of infection. In the authors' present experiments, C.F. antibody was demonstrated in all clinical or subclinical infections within 12～15 days after inoculation without exception. No continued long-period observations were made on puppies which had been subjected to virus inoculation without showing any signs of illness and no development of C. F. antibodies, so the authors have no data for a discussion whether surviving dogs turn to show positive C,F.T.

after 6 or 7 weeks or not.

It was very interesting that the inclusion bodies could not be demonstrated in C.F.T. positive puppies which were killed at 15~18 days after inoculation.

Recently the authors have isolated the above two strains of H.c.c. virus by the tissue culture technique following FIELDSTEEL, and are studying to analyse the antigenicity of cultivated materials.

SUMMARY

1. By using two strains, "YAMAGUCHI" and "MATSUDA", of H.c.c. which were encountered in Sapporo, serial transmission experiments on puppies aged 14~90 days were conducted. In case of the "MATSUDA" strain, the fatal infection could be transferred to the 3rd passage, however only non-fatal infection occurred in dogs at the 4th passage. On the other hand, in the "YAMAGUCHI" strain the authors could not bring out any fatal infections.

2. The clinical symptoms of puppies were variable even within the same litter. Fatal infection was found to occur in 28.6% (8/28) in the "MATSUDA" strain but 0% in the "YAMAGUCHI" strain. The total infection rates including fatal, severe, mild or inapparent form were 75% (21/28) with the former and 60% (6/10) with the latter strain.

3. Complement-fixing antibody was developed during the period of 12~15 days after inoculation. There seems to be no relationship among route, dose, severity of the clinical signs or viral strains examined.

4. In order to ascertain the prevalence of this disease in Japan, a survey on distribution of C.F. antibody among dogs from 4 districts was conducted. One hundred and eighty-six dog serum samples out of 374, nearly 50%, reacted positively. There seems not to be very much difference among districts (44~57%).

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