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CHARACTERISTICS AND PATHOGENECITY OF DUCK HEPATITIS VIRUS ISOLATED IN TAIWAN

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An acute lethal disease of ducklngs occurred in Taiwan from 1971 to December 1972. The disease was diagnosed for the first time as the duck virus hepatitis (DVH) by virological and pathological techniques.

Representative strains of the isolated virus, the Chiba strain of Japan, and the No. 5886 strain of the United States of America were pathogenic for duck kidney (DK) cell cultures as well as chicken embryos (CE). Plaque assay method, which had never been reported on a DVH virus, was proved to be applicable to the virus on DK cell culture.

Growth curve of the isolated virus on DK cell culture was examined. The virus was first recovered 3 and 4 hours after inoculation from cell phase and fluid phase, respectively. Virus titer showed logarithmic increase until 8 and 10 hours in respective phase, then changed to slow increase until it reached a maximum of about $10^{6.5}$ PFU/ml in both phases in 72 hours at which the experiment was terminated.

Eight-day-old ducklings were inoculated with the isolated virus intravenously and the virus titer of 20 organs in the ducklings was examined chronologically. The virus was first isolated from all organs 10-36 hours after inculation, and continued to be recovered from most organs until 144-216 hours. The virus was recovered for a longer period from the pancreas and cecum and shorter from the central nervous system. The virus disappeared from the serum within 43 hours after inoculation.

The neutralizing antibody of ducklings inoculated with the virus intravenously was detected 60 hours after inoculation. Antibody titer increased logarithmically until 66 hours, then changed to gradual increase until 120 hours reaching its peak of 2.5. It remained at this level up to 14 days after inoculation.

Fluorescent antigens were initially detected from liver 10 days and from spleen 3 days after inoculation. The antigens were detected from the thymus, pancreas, lung and rectum sometime between 10 to 22 hours after inoculation. No virus was detected from trachea, bursa of Fabricius, duodenum and cerebrum.

Histological examination of the liver of inoculated ducklings revealed necrosis of hepatic cells accompanied by mild heterophil infiltration or hemorrhage. These changes, together with those observed in field cases, however, did not seem specific for the disease.

Electron-microscopic observation of the hepatic cells in the inoculated ducklings detected degeneration of the cells and the presence of virus-like particles in the cytoplasm. The author, however, could not find any clear virus particle in this study.

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An acute infectious disease with high mortality was found at duck farms in duck production area, Hu-Nei town' in February 1971. The morbility, clinical signs, and histopathological observations were well studied. Virus was isolated from visceral organs of moribund or dead ducklings by using CE or DK cell cultures.

The disease spread rapidly over the island. The outbreaks were well investigated, many isolates were obtained from each individual case, and virus distribution in infected duckling was studied,

From the results of physicochemical properties of the isolate, nature of hemagglutination, and cross neutralization of the isolate with standard serum, the isolate was identified as duck hepatitis virus discribed by Levine & Fabricant (15).

Although duck cell cultures had been used to propagate DVH, many researchers could not employ the cell culture for virus titration. However, the duck kidney cell cultures were successfully applied for studies of growth curve in the present experiments.

Attempts were also made to determine the distribution of fluorescent antigen, vinus concentration in visceral organs, and histopathological changes in artiflcially infected ducklings.

MATERIALS AND METHOODS

1. Fertilized eggs

1) Chicken eggs: Eggs from chickens raised in isolate farm were used for virus isolation, virus titration, investigation of physicochemical properties of the virus, virus neutralization tests, and determination of virus growth curve.

2) SPF chicken eggs: SPE chicken eggs from the branch chicken farm of Japan National A nimal Health Institute were applied to prepare CEF using 9-11 day-old embryonated eggs, and to recover virus from artificially infected ducklings using 6-10 day-old embryonated eggs.

3) Duck eggs: Commearcial., 10 day-old duck embryos (DE) were used to propagate the virus.

2. Cell cultures

The technique described by Kawamura[14] was applied to prepare duck kidney cell cultures (DK) by using 3-6 week-old ducklings. Briefly, duck kidneys were aseptically collected, dispensed into flask by squeezing through syringe, suspended in 0.2% trypsin solution, about 1:20 kidney-trypsin in proportion, trypsinized for 10 minutes at 34-37°C. The supernatant of the first trypsinization was discarded while those of second, third and so on were harvested, centrifuged at 800 r. p. m. for 5 minutes, resuspended in YLE solution and stored at cold ice water. After 3-4 harvests, the trypsinized cells were spinned down at 800 r. p. m, for 5 minutes, resuspended in growth medium to make a 0.5% suspension (about 1.6×10^6 cells/ml), filtrated through a stainless net with 40 mesh, dispensed onto petri dish (5.5 cm in diameter, 5 ml/dish), and cultivated at 37:C.

Growth medium was Earle's solution supplemented with 0.5% lactoalbumin hydrolysate (Difco or BBL), 0.1% yeas-

telate (Difco), 5% fetal calf serum, 100/IU/ml penicillin, and 100 mg/ml streptomycin.

Three days after cultivation, the growth medium was replaced by maintenance medium in which 3% intead of 5% fetal calf serum was used. Confluent monolayer of DK culture should by formed and ready for use 4 days after cultivation.

3. Duck Hepatitis viruses

1) K418 isolate: Isolated by means of inoculation of embryonated chicken eggs from livers of moribund ducklings of the Kaoshung outbreak in April, 1971, and cloned twice by plaque method on DK cultures, and passed 6 times in embryonated chicken eggs (CE), then stored at -40°C. The virus titer was $10^{6.5}$ EID_{50}/ml in CE and $10^{6.0-6.4}$ PFU/ml in DK cultures.

2) Hsin-Chu isolate: Isolated by means of CE inoculation from livers of moribund ducklings of the Hsin-Chu outbreak in Sept., 1971. The infected allantoic fluid was harvested and stored at-40°C. The virus titer was $10^{6.25-6.75}$ EID_{50}/ml in CE and $10^{5.0-5.8}$ PFU/ml on DK cultures.

3) I-Lan isolate: Isolated by means of DE (duck embryo) inoculation from I-Lan case in March, 1973, cloned twice by plaque method, on DK cultures, passed in CE once and stored at-40°C. The virus titer was $10^{6.5-7.5}$ EID_{50}/ml in CE and $10^{5.4-6.0}$PFU/ml on DK cultures.

4) Chiba strain: The E-6 passage was obtained by courtesy of Dr. Szawa, Japan, in 1967. The virus was passed in CE once, harvested and lyophilized for storage. The virus titer was $10^{6.5-7.5}$ EID_{50}/ml in CE and $10^{6.5-6.8}$ PFU/ml on DK cultures.

5) No. 5886 strain: The E-83 passage was obtained by courtesy of Dr. Hwang, University of Pennsylvania, in 1972. The virus was cloned once by means of plaque mehtod on DK cultures and passed in CE once, then lyophilized for storage. The virus titer was $10^{7.5-8.9}$ EID_{50}/ml in CE and $10^{6.5-7.8}$ PFU/ml on DK cultures.

4, Hyperimmune serum

1) Preparation of immunization antigen: Virus was condensed to 1/50 of original volume by sealing in dialyzing tubes (Visking) and dipping in polyethylene glycol 6000 powder at cold room (4°C). The condensed virus was then dispensed in glass tubes and mixed vigorously with equal volume of Daifron, and centrifuged at 4,000 r. p. m. for 20 minutes. The supernatant was harvested, mixed with equal volume of Daifron, and centrifuged again. The harvest was inactivated with 0.2% formalin, and then mixed with equal volume of aluminum hydroxide (33 mg/ml). The inactivated antigen was used to immunize rabbits.

2) Immunization of rabbits: Large New Zealand White rabbits, body weight above 2.4 Kg, were used to prepare the hyperimmune sera. Two rabbits per isolate, each rabbit was intramuscularly inoculated with one ml of the antigen, and revaccinated one month later. The rabbits were bled one month after second vaccination. Sera were collected and stored in deep freezer. Before use, the sera were inactivated in 56°C for 30 minutes.

3) Preparation of hyperimmune sera in ducks: Two ducks, without DVH antibody, were used to prepare antisera

against the I-Lan the I-Lan isolate ($10^{7.0}$ EID_{50}/ml). One duck was orally inoculated with one ml of te virus at 8-week-old, 2 ml at 10-week-old, and 5 ml at 18-week-old. The other duck was intravenously inoculated with one ml of the virus at 8-week-old, intramuscularly inoculated with 2 ml of the virus at 10-week-old, and 5 ml at 18-week-old. Ten days after the last inoculation, the ducks were bled and sera were collected. The neutralization indexes of the sera were 5.3 and 4.8 respectively.

5. Virus isolation and titration

Lung, liver, spleen, kidney, trachea, and brain were collected from ducklings (moribund or dead) of each outbreak. Each organ was separately homogenized with the mortar pestle, diluted into ×5 or ×10 emulsion in YLE solution, frozen in -20°C overnight, thawed and then centrifuged at 3000 r. p.m for 20 minutes. The supernatant was harvested and used as inoculum.

1) Embryonated chicken egg inoculation: The embryonated chicken or duck eggs were inoculated with 0.1 ml of the preparations via allantoic cavity or yolk sac route. The eggs were candled every day for 7-9 days. Those died within 24 hours after inoculation were discaded, while those showed pathologic lesions, i. e. stunted embryo, edema, greenish liver, hemorrhge or necrosis in liver etc, were counted as infected. The 50% embryo infectious dose, EID_{50}, was calculated by means of Beherens-Kärber method.

2) Tissue culture inoculation: Confluent duck kidney cell cultures, about 4 day after cultivation, were inoculated with 4 ml of the emulsion, incubated at 37°C for 1 hour. The inoculum was replaced with 4 ml of maintenance YLE solution, the cutures were incubated 37°C, and observed for CPE for 48 hours,

Plaque method was applied to determine the virus titer. The DK cultures were inoculated with virus preparations, incubated at 37°C for 1 hour, added with 4 ml of first overlay and added again with second overlay 48 hours later. The plaque numbers were counted 24 hours after addition of second overlay.

The first overlay contained YLE maintenance solution, 7% 0.1 M tris buffer, 0.9% agar (Bacto agar or Difco), and 50 u/ml of mycostatin, while the second overlay was first overlay containing additional 8% of 0.1% neutral red solution.

6. Neutralization test

Both diluted virus constant serum and diluted serum constant virus methods were employed in the neutralization tests.

The diluted virus constant serum method. The virus, harvested allantoic fluid, was made serial 10 times dilutions, then each dilution mixed with equal volume of undiluted serum, incubated at 37°C for 1 hour; the other set of virus dilutions were mixed with equal volume of diluent and served as virus control. Each dilution was inoculated into several embryonated eggs. The neutralization antibody titer was expressed as log of the difference of infectious titer between the two sets of dilutions.

7. Fluorescent antibody technique

1) Preparation of frozen sections: Twenty ducks, 45-day-old, were intravenously inoculated with I-Lan strain of DHV $10^6 EID_{50}$ per duck. During 4 through 24 hours postinoculation, two ducks were killed every two hours, and visceral organs of the duck were harvested, immediately frozen in N-hexone, -70°C, then stored in-60°C freezer. The organs from two uninfected controls were also harvested and treated in the same way. The organs were made 4-6μ sections with cryostat, fixed with acetone for 5 minutes, and stored in -20°C freezer before FA staining.

2) Preparation of FA conjugate: Duck hyperimmune serum, with neutralization index $10^{5.3}$ and $10^{4.5}$, was diluted with equal volume of PBS, mixed with equal volume of saturated ammonium sulfate at 4°C for 30 minutes, centrifuged at 4000 r.p.m. for 5 minutes, the supernatant was discarded and the sediment was dissolved in PBS (two times of original serum volume), then repeated the precipitation procedures for 3 times. The final sediment was dissolved in PBS, ½ of original volumes), put into dialyzing tubes (Visking), dialyzed in running water and then against PBS in 4°C overnight. The solution was centrifuged at 3000 r. p. m. for 5 minutes, and the protein content was determined. Fluorescein isothiocyanate, 1/50 of total protein by weight, was solved in sodium bicarbonate buffer (pH 9.0), and added to the γ-globulin solution, mixed with magnetic stirrer at 37°C for 1 hour.

The mixture was passed through sephadex G-25 colume, eguilibrium in 0.005 M K_2HPO_4 solution, in order to elute the unlabelled fluorescent dye. The conjugate was then passed through DEAE cellulose colume to increase the FA specificity. The conjugate was added with 0.1% of sodium azide and stored in refrigerator before use.

8. Virus replication in embryonated chicken eggs

The DVH isolate was inoculated via allantoic sac into each 40 embryonated eggs, 7-day-old and 10-day-old. Allantoic fluids were harvested from each 2 eggs at 3, 6, 9, 12, 18. 24, 32, and 48 hours postinoculation, respectively. The virus concentration in the harvests was titrated on DK cultures by plaque method, and the growth curve of the isolate was determined.

9. Virus distribution and antibody survey on DHV infected ducks

1) Virus distribution: Sixty-four commercial ducklings, 8-day-old, were intravenously inoculated with DHV I-Lan strain, $10^{6.0}$ EID_{50} per duck. Twenty tissues were harvested from infected ducklings at different intervals postiuoculation, and stored at -45°C before being subjected to virus titration.

2) Histopathological observation: Twenty-one SPF Peking ducklings, 8-day-old, were separated into two groups. Eighteen of them were orally inoculated with I-Lan isolate, 0.5 ml per duck and $10^{7.7}$ EID_{50}/ml. and the other 3 were served as uninfected controls.

Each two infected ducklings were killed and examined at 16 and 24 hours PI, respectively. Four of the inoculated ducklings died 25 hours PI, and 10 of them died after 40 hours PI. All the ducklings were examined, and livers

were harvested for histological observation (HE stain). Some of the livers were also subjected to virus isolation.

Twenty-two commercial Peking ducklings, 17-day-old, were purchased from Osaka market and raised up to 45-day-old, then intravenously inoculated with I-Lan isolate, $10^{7.6}$ EID_{50}per duck. During 4 through 24 hours PI, two ducks were killed and examined every two hours, and livers of the duck were subjected to histopathological examination; those from control ducks were also included.

3) Electron microscopy observation: Livers, 5 cubic mm in size, from DHV inoculated duckling were collected, fixed with 2.5% glutaraldyhyde at 4°C for 10 minutes, fixed in 1% OsO_4 for 2 hours, dehydrated, embedded in Epon 812, then subjected to thin section, double stained with uranyl acetate and lead citrate, and examined with electron microscope (Hitach HS-9 type).

4) Baby geese inoculation test: Two baby geese, 3-day-old, were used for the test, one intramuscularly inoculated with $10^{6.0}$ EID_{50} Hsing-Tsu isolate, the other intracerebrally inoculated with 0.03 ml ($10^{6.5}EID_{50}$/ml) of the virus. The geese were observed for clinical signs and examined after death.

RESULT

1. Epidemiological investigation

1) Epidemiology

An acute with high mortality disease, duck viral hepatitis, was observed among broiler ducklings at Hu-Nei, Kaoshiong, in February 1971.

The outbreak was noted in 58 out of 72 farms, i. e. 80% of the farms in Hu-Nei area. The mortality among the farms was ranged 2-85% with an average of 29% (41640 out of total 142180 ducklings).

The disease was then found at other areas in Kanshiong, Pintong and Taitong couuties. in a short period of time, the disease spread toward central and northern parts of the island, i.e. Tainan, Chia-I, Yun-lin, Changhwa, Taichung, Hsin-Tsu, Taipei, I-Lan, and Taoyuen counties. The disease was not under well controlled until December 1972. The total loss was over 2 million ducklings. Sporadic cases were also noted at I-Lan in March, 1973, and at I-Lan, Kaoshiong and Hsin-Tsu counties in 1978.

2) Clinical signs

Clinical cases were only found in ducklings under 2-week-old, and no cllnical sign was seen in ducklings over 3-week-old. The main signs were depression, anorexia, ruffled feather, ataxia, struggled movement and partially closed eyes. The affected ducklings fell on their sides, kicked spasmodically with legs or wings, and died suddenly with head drawn back. Vomiting and watery or greenish diarrhea were also noted ocassionally.

3) Pathological findings

The gross lesion was mainly found in liver. The affected liver was slight or moderate swelling and yellowish-brown in color with pinpoint hemorrhage or scattered petechia (Fig. 1). Ocassionally, kidney swelling and hemorrhage, or cerebral meninge congestion were also observed.

Fig. 1. Gross lesions of liver with scattered petechiae in naturally infected case (Hu-Nei, Kaoshiong)

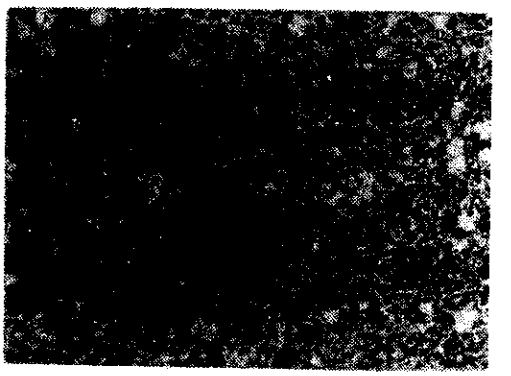


Fig. 2. Microscopic lesions of liver, with necrosis of hepatic cells, in naturally infected case. HE stain, ×250

4) Histopathological findings

The findings were summarized in Table 1.

Table 1. Histopathological findings in field cases

Cases	Liver		Reticuloendothelial Cells Proliferation of spleen	Lung Edema	Inflammation of Proventriculum	Myositis
	Degeneration	Necrosis				
No. 1	##	+	+	+	−	−
No. 2	##	##	+	+	−	−
No. 3	##	+	+	+	+	+

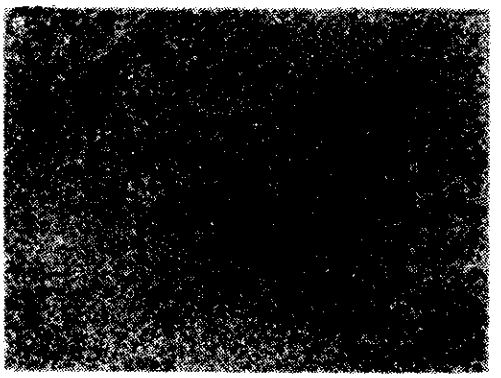
Remark: ##, markedly; #, moderately; +, slightly; −, negative.

2. Virus isolation

Twelve isolates were obtained from the field cases since the Hu-Hei outbrek in April, 1971 (Table 2).

Table 2. Virus isolated from field Cases in Taiwan

Cases	Time of outbreak	Place of outbreak	Age of duckling (Days)	Designate	Culture method
1	April 1971	Kaoshiong	10	K 417	CE and DK
2	April 1971	Kaoshiong	8	K 418	CE and DK
3	April 1971	Kaoshiong	12	K 420	DK
4	April 1971	Kaoshiong	3	K 421	CE and DK
5	June 1971	Taipei	8	Taipei	CE and DK
6	Sept. 1971	Chia-I	6	Chia-I	CE and DK
7	Sept. 1971	Yunlin	11	Yunlin	CE and DK
8	Sept. 1971	Hsin-Tsu	10	Hsin-Tsu	CE and DK
9	Oct. 1971	I-Lan	5	Lou Tong	CE and DK
10	March 1972	Pingtong	12	Pingtong	CE and DK
11	April 1972	Taitong	12	Taitong	CE and DK
12	March 1973	I-Lan	10	I-Lan	DE and DK



Pig. 3. Microscopic lesions of spleen, with epithelial cell proliferation and atrophic lymphoid tissue around the central arteriole, in naturally infected case. HE stain, ×100

1) Virus isolation in embryonated eggs

Homogenized tissue emulsion of infected duckling was inoculated via allantoic cavity or yolk sac into embryonated eggs (6-7 days old). The eggs were observed every days. Most of the eggs died within 2-6 days after inoculation. The infected embryos showed stunted embryos with abdominal edema, greenish liver, hemorrhage and necrosis.

2) Virus isolation in duck kidney cell cultures.

Homogenized tissue emulsions of infected ducklings were inoculated onto DK cultures. Cytopathic effects were noted 24 hours after inoculation. Cell rounding and then peel off. The plaques were oval or semilunar in shape. Viruses were rlease into the culture media. The plaque method could be applied for virus titration.

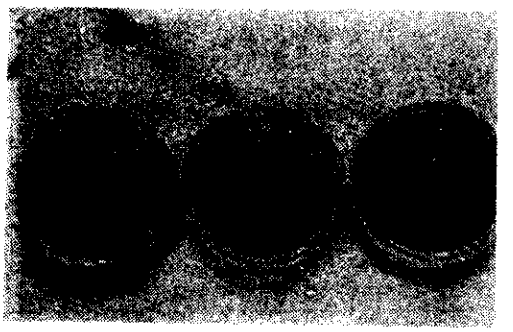


Fig. 4. Plaque formation of DHV on DK cultures. (3 days after inoculation)

3) Virus titers of the isolates

The isolated viruses, after 1 or 2 passages in CE, had titers of bout $10^{5.8-6.5}$ EID_{50}/ml or $10^{5.9-6.5}$PFU/ml. (Table 3)

Table 3. Virus titer of the isolates in CE or DK cultures

Cases	Isolation Uesignate	Passage	EID_{50}/ml **	PFU/ml **
1	K 417	CE-2	6.3	6.3
2	K 418	CE-2	6.5	6.4
3	K 420	DK-1	6.0	5.9
4	K 421	CE-2	6.0	6.1
6	Chia-I	CE-1	6.0	6.2
7	Yunlin	CE-1	5.8	5.9
8	Hsin-Tsu	CE-1	6.5	6.3
9	Loutong	CE-1	6.5	6.4
12	I-Lan	CE-1	6.5	6.5
	Chiba*[1]	CE-7	6.5	6.3

* DVH control virus ** Expressed as logarithm 10

4) Virus distribution in infected ducklings of field cases

Homogenized tissue emulsions of organs from ducklings of field cases were titrated in CE or on DK cultures. The results were summarized in Table 4.

Table 4. Virus Distribution in Infected Ducklings Titrated by Chicken Embryos (CE) and Duck Kidney (DK) Cells

Organs	Case 2		Case 3	
	CE	DK	CE	DK
Trachea	4.0*	3.2**	4.0	3.6
Lung	⩾4.5	5.6	5.8	5.5
Air sac	NT	NT	0	3.5
Heart	NT	NT	4.0	3.8
Liver	⩾4.5	5.9	5.8	4.5
Bile	NT	NT	6.0	5.6
Spleen	4.5	5.7	6.8	5.7
Kidney	⩾4.5	5.4	6.5	
Pancreas	NT	NT	8.5	6.8
Cerebrum	4.0	3.1	0	0
Cerebellum	NT	NT	0	0
Spinal cord	NT	NT	0	2.4
Esophagus	NT	NT	5.0	4.7
Proventriculus	NT	NT	5.5	4.4
Duodenum	⩾4.5	5.7	7.3	5.7
Cecum	NT	NT	8.5	6.1
Rectum	NT	NT	5.8	5.9
Bursa	NT	NT	2.0	3.5
Muscle	NT	NT	4.0	3.2

* Log_{10} EID_{50}/ml ** Log_{10} PFU/ml

3. Physicochemical properties of the isolates

The results were showed in Tables 5 and 6.

Table 5. Physicochemical properties of the isolate

Virus	Isolate Designate	Untreated	Treatment				
			0.1% ethylether	0.1% SDC	0.2% Trypsin	pH 3.0	pH 9.0
DVH Isolate	K 418	5.0*	4.8	5.0	5.0	4.3	4.5
Avian Influenza	Tansui	7.5	0	0	⩾7.5	3.3	5.5
Newcastle Disease	Sato	5.5	0.5	0.5	6.8	2.8	5.5

* Log_{10} EID_{50}ml in embryonated chicken eggs

Table 6. Heat Resistance of the isolate

Virus	Isolste Designate	Untreated	56°C 30 min	
			1M $MgCl_2$	Distilled water
DVH	K 418	5.0	4.5	3.0
Avian Influenza	Tansui	7.5	4.3	3.5
Newcastle Disease	Sato	9.5	4 3	0

The results indicated that the DVH isolate was resistant to the treatment of 0.1% ethylether, 0.1% sodium deoxycholate (SDC), 0.2% trypsin, pH 3.0, pH 9.0 and heat.

The virus titer was unaffected when passed through mllipore filter sized 220 nm or 100 nm, dropped a little while passed through filter sized 50 nm, and lost completely as filtrated with 10 nm filter.

Table 7 indicates the virus titer in emulsion of various organs after 7 years' storage in a -20°C freezer.

Table 7. Storage tests

Period of Storage	Emulsion of Organs						
	Trachea	Lung	Liver	Spleen	Kidney	Intestine	Brain
Origin	3.2*	5.6	5.9	5.7	5.4	5.7	3.1
7 years	0	3.9	3.6	1.4	4.0	4 6	0

* Log_{10} PFU/ml In DK

4. Hemagglutination of the virus

Hemagglutination tests were carried out at room temperature and 4°C by using red blood cells from chicken, duck goose, pigeon, horse, yellow cattle, cow, pig, sheep, rabbit, guinea pig, and mouse. No hemagglutination of the isolate K418 was noted from the tests.

5. Cross neutralization tests

The neutralization antibody titers among the DVH strains were variable (Table 8).

Table 8. Cross neutralization tests among DVH strains

Virus / Ab	K 418	Hsin-Tsu	Chiba	No. 5886
K 418	5.2*	4.1	4.1	5.5
Hsin-Tsu	1.2	1.5	1.9	2.4
Chiba	3.8	3.0	3.7	3 3
No. 5886	3 8	3.1	3.1	3.5

* Neutralization Index

6. Growth curves of the virus

1) Growth curve of K 418 in DK cultures

The virus appeared in the cultured cells 3 hours after inoculation, increased geometrically within 8 hours PI, and reached $10^{5.4}$ PFU/ml 72 hours PI. The virus started to release from the cells to the media at 4 hours PI, reached at $10^{4.1}$ PFU/ml at 10 hours PI and $10^{6.6}$

PFU/ml at 72 hours PI(Fig. 5).

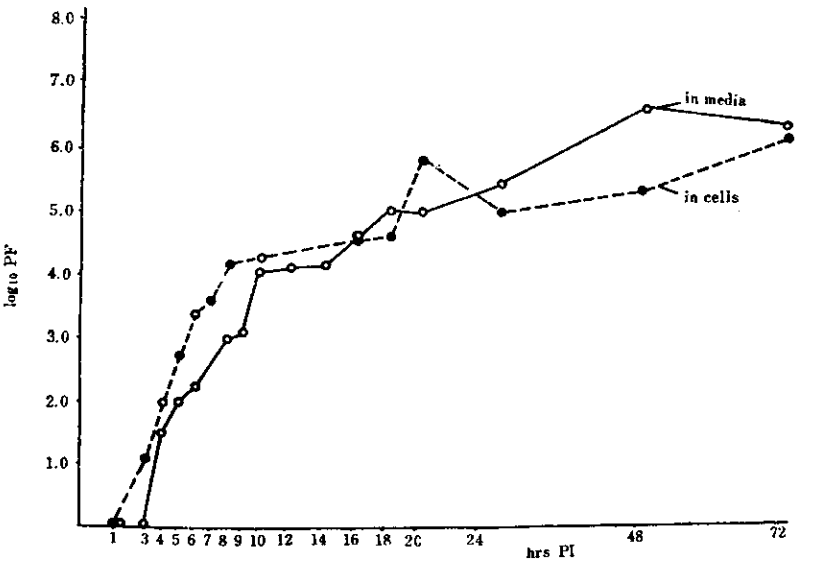


Fig. 5. Growth curves of DVH K418 in DK cultures

2) Growth curve of I-Lan isolate in 7-day-old embryonated chicken eggs:

Twelve hours after inoculation, the virus titer in allantoic fluid, CAM, or embryo of the inoculated eggs started to incease. The virus titer reached peak at 48 hours PI, and then dropped a little afterward (fig. 6).

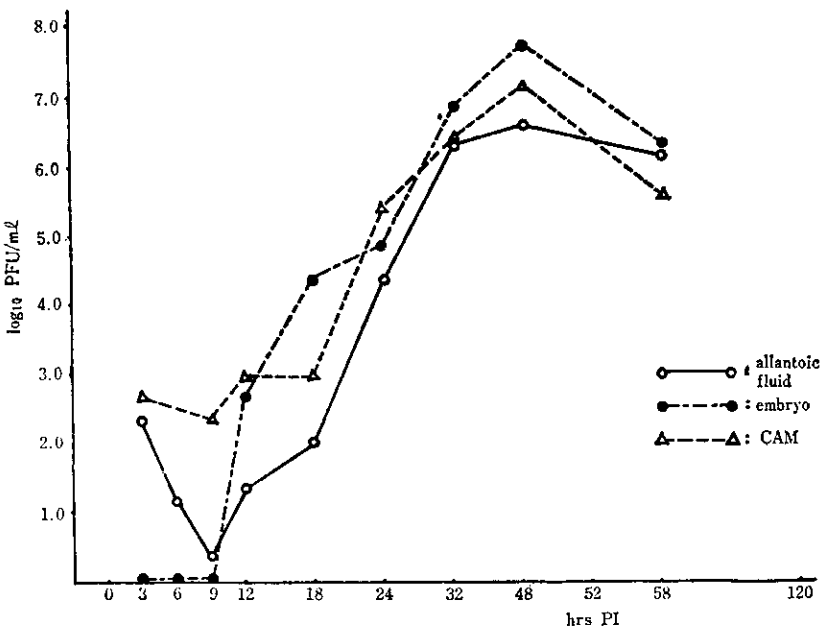


Fig. 6. Growth curves of I-Lan isolate in 7-day-old embryonated chicken eggs.

3) Growth curves of I-Lan isolate in 7-day-old embryonated chicken eggs

Figure 7 showed that the virus grew rapidly at 9-12 hours PI, reached peak in CAM at 24 hours PI, in embryo and allantoic fluid at 52 hours PI.

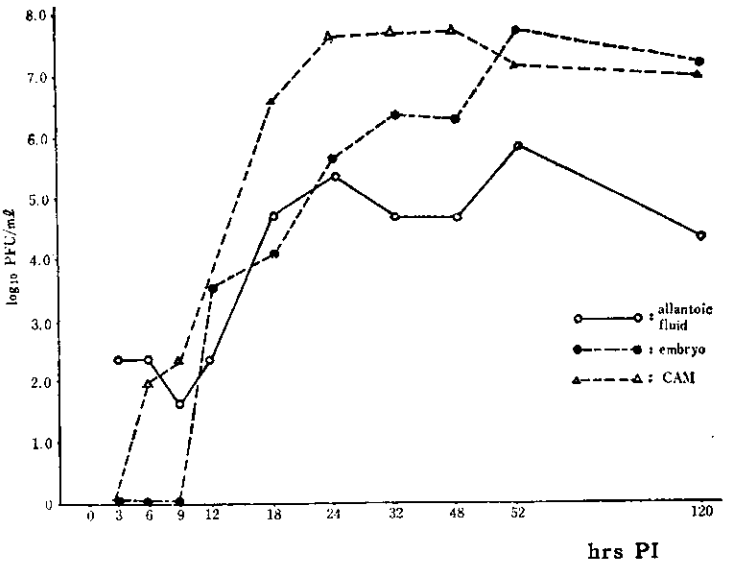


Fig. 7. Growth curves of the I-Lan isolate in 10-day-old embryonated chicken eggs.

Table 9. Virus, I-Lan Isolate, Distribution in Inoculated Ducklings

Organs	First isolation hrs PI*1	Persistant Infection hrs *2	Peak Titer *3	Peak hrs PI *4	No. Virus Isolated hrs PI *5	Last Isolation hrs PI *6
Trachea	18		4.6	48	144~192	192
Lung	≤10		5.6	48	144	144
Air sac	≤10		6.2	30	144	144
Heart	18		4.6	42	144	144
Liver	≤10	216	7.8	18		216
Bile	≤10	192	7.0	14		192
Spleen	≤10	216	6.6	30		216
Kidney	≤10	216	6.8	42		216
Pancreas	14	264	8.4	66		264
Cerebrum	36		3.1	66	120~132	132
Cerebellum	14		3.5	144	114~144	144
Spine	24		4.4	48		96
Esophagus	14		6.3	48	120~132	132
Proventriculus	≤10		5.2	36	144~192	192
Duodenum	≤10		6.7	24	168~216	216
Cecum	≤10	264	6.1	144		264
Rectum	14	216	6.2	42		216
Bursa	14		5.4	14		108
Muscle	14	216*7	4.5	60		213
Serum	18		4.7	36		48

Remarks: 1. 10 indicated that virus could be detected at 10 hours PI.
2. The time made last virus isolation.
3. Log_{10}PFU/ml.
4. The time for virus titer to reach the peak.
5. The period of time that virus could not be detected.
6. The number with underline indicated that virus could not be detected.
7. The virus could not detected at 114 and 168 hours PI.

7. Virus distribution in artificially inoculated ducklings

The virus could be detected at 10 hours PI in lung, air sac, liver, bile, spleen, proventriculus, duodenum and cecum, 14 hours PI in pancreas, cerebellum, esophagus, rectum, bursa and muscle, 18 hours PI in trachea, heart and serum, 24 hours PI in spine, and 36 hours PI in cerebrum, i. e. the virus could distribute to all organs and body fluid. The virus could be reisolated from the inoculated ducklings until 192-264 hours PI in liver, bile, spleen, kidney. pancreas, cecum, rectum and muscle.

The virus titers in various organs ranged in 3.1--8.4 (log_{10}PFU/g). The virus titer reached peak level in bile as early as 14 hours PI and in liver 18 hours PI. No virus could be isolated in the end of the experiment, 336 hours PI.

2) Antibody response in artificially inoculated ducklings

Sera collected at various intervals were subjected to serum neutralization tests in CE. The results were summarized in Fig. 8.

The neutralization antibody could be detected 60 hours PI (neutralization index 0.7). The antibody level rose rapidly at 66 hours PI, NI 1.6, reached peak at 120 hours PI, NI 2.5, and maintained at the level of NI 1.7-2.5 up to 336 hours PI.

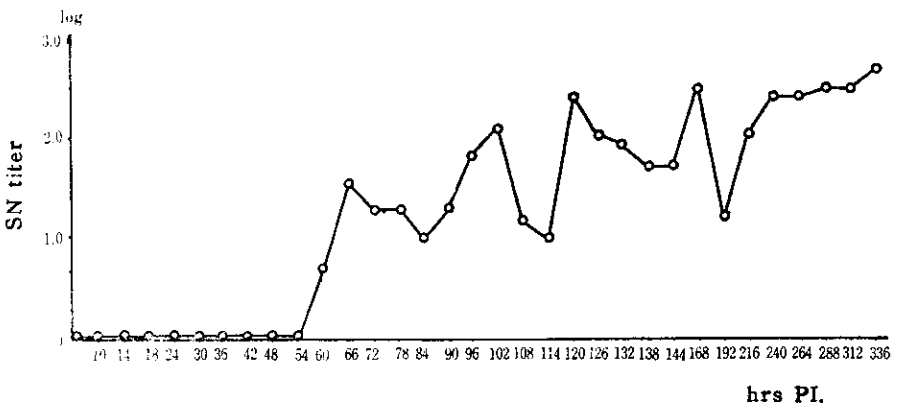


Fig. 8. Serum neutralization antibody response in ducklings inoculated with DVH, I-Lan strain.

3) Detection of antigen distribution by means of fluorescent antibody technique

Various organs were collected for FA detection at 2 hours intervals, started at 4 hours PI. Scattered fluorescent cells or patched FA pancreas could be observed in one case of liver at 10, 12, 14 hours PI, and in livers of most cases 24 hours PI.

In red pulp of spleen, the specific fluorescence could be found in one case 8 hours PI, and in most cases 24 hours PI. The results were summarized in Table 10. and Fig. 9.

Table 10. Detection of virus Distribution in Ducklings Inoculated with I-Lan Isolate by Means of Fluorecent Antibody Technique

PI (hrs)	Organs / Puck No.	Trachea	Thymus	Lung	Liver	Spleen	Bursa	Pancreas	Duodenum	Rectum	Cerebrum
0	17268	—*	—	—	—	—	—	—	—	—	—
	17269	—	—	—	—	—	—	—	—	—	—
4	17270	—	—	—	—	—	—	—	—	—	—
	17271	—	—	—	—	—	—	—	—	—	—
6	17272	—	—	—	—	—	—	—	—	—	—
	17273	—	—	—	—	—	—	—	—	—	—
8	17274	—	—	—	—	—	—	—	—	—	—
	17275	—	—	—	—	+	—	—	—	—	—
10	17276	—	+	—	+	++	—	—	—	—	—
	17277	—	—	—	—	+	—	—	—	—	—
12	17278	—	—	—	—	++	—	—	—	—	—
	17279	—	+	—	+	—	—	—	—	—	—
14	17280	—	++	—	+	+++	—	—	—	—	—
	17221	—	+	—	—	+++	—	—	—	—	—
16	17282	—	—	—	+	++	—	—	—	—	—
	17283	—	—	—	+	++	—	—	—	—	—
18	17284	—	—	—	++	+++	—	—	—	—	—
	17285	—	—	—	+	+	—	+	—	—	—
20	17286	—	—	—	+	+	—	—	—	—	—
22	17287	—	—	++	+	+	—	—	—	—	—
	17288	—	—	—	++	+++	—	+++	—	+	—
24	17289	—	—	—	+++	++	—	—	—	—	—

Re8marks: Intensity of fluorescence observed
—: negative, +: weak, ++: moderate +++: strong

Fig 9. Specific fluorescence was noted in red pulp of spleen 14 hours PI. ×600.

4) Pathological changes in ducklings inoculated with I-Lan isolate

a) Trial 1: SPF ducklings of 8-day-old were orally inoculated with DVH I-Lan isolate. Four of the inoculated ducks died in 1 day PI, and the other died within 2 days PI, The clinical signs observed were depression, anorexia, ataxia and shaking head. The virus could be recovered from the inoculated ducklings with virus titer of $10^{4.75-6.5}$ EID_{50}/ml while no virus was found in the uninoculated controls (Table 11).

Table 11. Virus Recovery from the Ducklings Inoculated with I-Lan Isolate

Group	Duck No.	PI (hrs)	Condition	Virus Titer In liver
Control	17135	0	killed	<1
	17136	0	killed	<1
	17154	48	killed	<1
Inoculated	17137	16	killed	4.75
	17138	16	killed	6.25
	17142	24	killed	6.5
	17143	24	killed	6.5
	17145	25	died	6.5
	17147	25	died	5.5
	17155	40	died	6.0
	17156	40	died	5.25

b) Trial 2: Commercial ducks of 45-day-old were intravenously inocualted with DVH I-Lan isolate. The clinical signs were observed. Various organs were collected for virus recovery and histopathological examination.

c) Gross lesions

1) Trial 1: The results were summarized in Table 12. Four ducks killed at 16 hours or 24 hours PI showed liver swelling and decoloration; liver hemorrhage in 2 ducklings, spleen swelling in 2 ducklings, kidney hemorrhage in 1 duckling, and rhinitis in all of them.

Fourteen ducks died within 2 days

Table 12. Gross Lesions of SPF Ducklings Orally Inoculated with I-Lan Isolate.

Group		PI (hrs)	Liver			Spleen swelling	Kidney hemo.	Bursa swelling	Rhinitis	Lung edema
			Swelling	Docoloration	Hemorrhage					
Control	17135	—	—	—	—	—	—	—	—	—
	17136	—	—	—	—	—	—	—	—	—
	17152	—	—	—	—	—	—	—	—	—
Killed	17137	16	+	+A	—	—	—	—	+	—
	17138	16	+	+A	—	—	—	—	+	—
	17142	24	++	+A	+	+	—	—	+	—
	17143	24	++	+A	+	+	+	—	+	—
Died	17145	25	++	+B	##	—	++	—	++	+
	17146	25	—	+A	+	—	++	—	+	##
	17147	25	++	+C	++	—	##	—	+	—
	17148	25	—	+A	++	—	++	—	+	+
	17155	40	##	+C	##	+	++	++	+	—
	17156	40	++	+B	+	+	++	##	+	+
	17157	40	++	+B	++	+	++	+	+	+
	17158	40	++	+B	+	++	++	+	+	+
	17159	40	+	+A	##	+	++	+	+	+
	17160	46	++	+B	++	—	##	+	+	+
	17161	40	++	+B	++	—	++	+	+	+
	17162	40	##	+A	—	—	++	##	+	++
	17163	40	++	+B	+	—	##	+	+	+
	17164	40	++	+B	+	++	++	+	+	++

Remarks: —: negative, +: slight, ++: moderate, ##: marked
*A: decoloratisn, B: congestion, C: severe congestion

PI and showed liver swelling and decoloration with mild or marked hemorrhage, kidney hemorrhage, rhinitis, spleen swelling (6 out of 14 ducklings), bursa swelling (10 out of 14 ducklings), lung edema (12 out of 14 ducklings). No pathological lesion was observed in killed control ducks.

2) Trial 2: The only one duckling showed gross lesions was the one died at 24 hours PI. No pathological change was observed in the other 19 infected ducks killed at different times PI. The gross lesions were liver mild swelling and slight decoloration with pin-point to miliary hemorrhage, spleen mild swelling, kidney slight swelling with scattered pin-point hemorrhage.

d) Histopathological findings in livers of ducklings inoculated with DVH I-Lan isolate

The liver lesions observed in trial 1 were similar to those of trial 2. Hepatic cell necrosis with a few granulocytes infiltration was noted. Hepatic cells with eosinophilic cytoplasm, hemorrhage with focal or diffuse necrotic areas were found all over the liver (13 and 14).

Table 18. Histopathological Lesions in Livers of Ducklings Inoculated with I-Lan Isolate (Trial 1)

	Duck No.	Hepatic Cell with Eosinophilic Cytoplasm	Hepatic Cell Lysis	Hepatic Cell Necrosis	Granulocyte Infiltration	Hemorrhage	Reticulum Cell Activation	Congestion
Control Killed	17135	—	—	—	—	—	—	—
	17136	—	—	—	—	—	—	—
	17154	—	—	—	—	—	—	—
Killed	17137	—	—	—	—	—	—	—
	17138	—	—	—	—	—	—	—
	17142	##	#-	#-	+	—	—	+*
	17143	##	#-	#-	+	—	—	+*
Infected Died	17145	##	##	##	+	—	—	##*
	17146	##	##	##	+	—	—	##*
	17147	##	##	##	+	—	—	##*
	17148	##	##	##	+	—	—	##*
	17155	##	##	##	+	—	—	##*
	17156	##	##	##	#-	—	—	##*
	17157	##	##	##	+	—	—	##*
	17158	##	##	##	+	—	—	##*
	17159	##	##	##	+	—	—	##*
	17160	##	##	##	+	—	—	##*
	17161	##	##	##	+	—	—	##*
	17162	##	##	##	+	—	—	##*
	17163	##	##	##	+	—	—	##*
	17164	##	##	##	+	—	—	##*

Remarks: —: negative, +: mild, #-: moderate, ##: marked.
* hemorrhage.

Table 14. Histopathological Lesions in Livers of Dnck Inoculated with I-Lan Isolate (Trial 2)

	Duck No.	PI (hrs)	Hepatic cell: Eosinophilic cytoplasm	Hepatic cell: Lysis	Hepatic cell: Necrosis	Granulocyte Infiltration	Activation of Reticulum Cells	Congestion
Control	* 17268	—	—	—	—	—	—	—
Control	* 17269	—	—	—	—	—	—	—
Infected	17270	4	—	—	—	—	—	—
Infected	*1 17271	4	—	—	—	—	—	+
Infected	17272	6	—	—	—	—	—	—
Infected	17273	6	—	—	—	—	—	—
Infected	17274	8	—	—	—	—	—	—
Infected	17275	8	—	—	—	—	—	—
Infected	17276	10	—	—	—	—	—	—
Infected	17277	10	—	—	—	—	—	—
Infected	17278	12	—	—	—	—	—	—
Infected	17279	12	+	—	+	+	—	—
Infected	17280	14	—	—	—	—	—	—
Infected	17281	14	—	—	—	—	—	—
Infected	17282	16	+	—	+	+	—	—
Infected	17283	12	++	+	+	+	+	—
Infected	17284	18	++	+	++	+	—	+
Infected	17285	18	+	—	—	+	—	—
Infected	17286	20	+++	—	++	+	+	—
Infected	17287	22	—	—	+	+	—	—
Infected	17288	22	+++	—	++	+	+	—*2
Infected	*1 17289	24	+++	+++	+++	+	+	+++*2

Remarks: —: negtive, +: mild. ++: moderate, +++: severe lesions
* Control
*1 Died
*2 Hemorrhage

e) Electron microscopic observation of livers from ducklings inoculated with I-Lan isolate:

Liver from duck no. 17137 killed at 16 hours PI was subjected to electron microscopic examination. The fine structures of the hepatic cells were found no particular change. There were areas of high electron density granules in hepatic cell cytoplasm, glyconucleotide granules attached to the rough surface of vacuoles. Liver from duck no. 17288 showed large lipid droplets in cytoplasm of hepatic cells. The fine organelles in cytoplasm disappeared and lysed. Swelled mitochondria, vacuoles, glyconucleotides were noted in the cytoplasm of cell debris. Swelled glyconucleotides and granules with high electron density, sized 30-50 nm were also observed in some cytoplasms (Fig. 10).

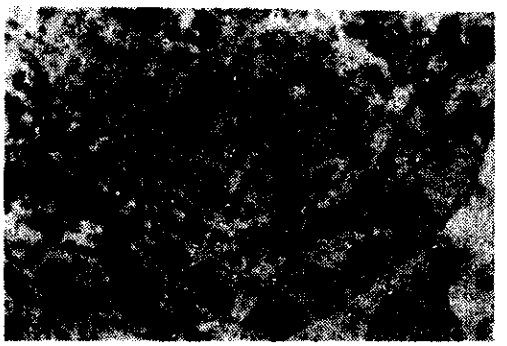


Fig. 10: Electron microscopic findings of liver from duck no, 17288. Irregular granules with high electron density and viruslike particles were found in the cytoplasm of degenerated cells. Embedded with Epon 812. double stained with uranyl acetate and lead. x22,000.

f) Geese and chicks inoculated with DVH isolate, Hsin-Tsu strain:

Geese of 3-day-old were intramuscularly or intracerebrally inoculated with DHV, Hsin-Tsu isolate. Clinical signs, observed 4 days PI, were shaking head, sitting, depression and trembling. The intramuscularly inoculated goose died 15 days PI, while that intracerebrally inoculated one died 18 days P. I. Decolorated liver with petechia, swelled kidney with hemorrhage and congested meninge were found in those geese. During two weeks' observation, no clinical sign was noted in any chick subcutaneously, intramuscularly, intranasally, intranasally or intracerebrally inoculated with DVH, Hsin-Tsu isolate.

DISCUSSION

The virus, isolated in CE and DK cultures from an outbreak of ducklings with high mortality, was first identified to be duck hepatitis virus in April, 1972. Not any duck disease with such a high mortality was noted besides fowl cholera. It was suggested that the virus originated from the imported breeding ducks or eggs although no direct evidence was found.

Levine and Farbricant [15] indicated that duck hepatitis virus could be cultivated and titrated in embryonated chicken eggs (CE) while the others reported that the virus might not grow in chicken embryo fibroblast, chicken embryo liver, chicken kidney, or duck embryo liver cell cultures[13,20,21].

Fitzgerald et al. [7] and Mailboroda [16] described that DHV, after 9-26 passages, could induce CPE in duck embryo kidney cultures. Sazawa et al [20] observed unclear plaque of DHV on duck embryo fibroblast cultures and implied that the DEF system might be used to determine the virus titer. On the other hand, Hwang [16] could not find any plaque of DHV on DEF cultures. Mailboroda [17] could detect virus concentration in cell cultures by means of FA technique. Although there were so many investigations on growth of DHV in tissue cultures, the tissue culture system was not widely applied to the DHV studies.

In this study, the embryonated chicken eggs (CE) and duck kidney cell cultures were applied to isolate DHV from the field case. CPE and plaque formation were observed on the DK cultures. The DK culture system was also employed to determine the virus titer, to detect the virus distribution in inoculated and naturally infected ducklings. The DK culture system was proved to be better than CE inoculation.

By the cross neutralization tests with CHIBA and No. 5886 DHV strains, and also by determination of physical-

chemical properties, the K418 and Hsing-Tsu isolates were proved to be DHV. Generally, there was no antigenic difference among the strains, however. Asplin [6,7] and Toth [22] described that some DHV isolates were not serologically related. Further studies were needed to determine whether there were different serotypes among the DHV isolates or not.

Hwang [9] investigated the distribution of virulent or attenuated DHV in lung, heart, liver, spleen, kidney, and muscle of inoculated ducklings, and found the virus concentration in these organs were about $10^{3.7-6.4}EID_{50}$/ml. Besides, Akulov [2] also described the virus distribution in inoculated ducklings and geese. In the present studies, systematic and chronogical analysis of virus distribution in inoculated or naturally infected ducklings was made. It was found that the prompt growth of the virus in almost organs might be the cause of sudden death.

The virus could be continuously detected in intestine of infected ducklings as long as 264 hours PI, and this indicated its nature of Enterovirus. Shed virus in feces might be one of the most important source of infection.

Viremia was noted during 18-48 hours PI and virus disppeared gradually from blood circulation 60 PI when serum neutralization antibody was detected. Similar reports were also found by Friend and Trainer [8] and Hwang [11].

FA technique was used for DVH diagnosis by Vertinskii et al [23]. In this study, specific fluorescenee was found in spleen 8 hours PI, in liver 10 hours PI, and in most of organs 24 hours PI. It was concluded that FA technique was good for early diagnosis of the disease.

Hundred percent mortality was found in SPF ducklings inoculated with DHV at 8 days of age, while negligible mortality (one out of 20) in infected 45-day-old ducks. The younger the ducklings the higher the mortality was.

The microscopic lesions of livers from moribund or dead ducklings in artificial inoculation trials were similar to the findings described by Fabricant et al [4]. Necrotic cells with eosinophilic stain were found 12 hours PI, liver swelling with cell degeneration and diffuse necrotic areas were observed 16-22 hours PI, then hemorrhage and focal necrosis were noted 24 hours PI. Mild proliferation of bile ducts was also found in an infected dead ducklings. The various degrees of histopathological changes might be depended on the susceptibility of ducklings and virulence of DHV used in the trials.

The electron microscopic changes in hepatic cells were similar to those described by Richter [18] and Aimiker [1]. In hepatic cell cytoplasm, there were expanded vesicles, granules, lysed debris, high electron density particles, sized 0.3-0.6μ, swelling glyconucleotides, and some virus-like particles, sized 30-50nm. The results that DVH isolates could infect young geese but not chicks were the same as those described in other reports [2,3,12,20].

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臺灣之鴨肝炎病毒性及病原性

呂 榮 修

中 文 摘 要

1972 年 2 月～1972 年 12 月間，著者對於臺灣發生之小鴨急性致死性疾病，從病毒學及組織病理學的研究，首次確證本省發生鴨肝炎。

本省分離之病毒與日本千葉株及美國 No 5886 株等均可對鷄胚（CE）顯示有病原性，對鴨腎（DK）細胞亦有病原性。著者並確立過去從未見於報告的利用 DK 細胞的斑點定量法。

分離的病毒經接種於 DK 細胞後所檢查的結果，在 3～4 小時後起，各檢出液相及細胞相病毒，8～10 小時示對數的增殖病毒量達 $10^{4.0}$PFU/ml，並持續增殖至 72 小時，最高值約達 $10^{9.5}$PFU/ml。

以分離的病毒靜脈接種於 8 日齡小鴨時，接種後 10～36 小時起可自體內 20 個部位檢出病毒，並在體內各部持續 144～216 小時之久。由胰臟及盲腸可較長期的分離到病毒，但由中樞神經則較短，而由血清中亦僅可於 48 小時內檢出病毒。

人工感染鴨病例體內的中和抗體的出現情形爲於靜脈接種後 60 小時開始可檢出，然後急激增加（1.6）至 66 小時，此後即變爲徐緩至 120 小時（2.5）而保持中和價 2.5 左右至 240～336 小時。

人工感染病例時，肝自接種後 10 小時及脾則自 8 小時起至 24 小時內，可檢出螢光抗原。而自胸腺、胰臟、肺及直腸等則均在 10～22 小時內的任何時間，確認其螢光抗原的存在。但自氣管、滑氏囊、十二指腸及大腦等部位則未曾檢出。

人工接種病例的肝臟病理組織檢查中，顯示帶有僞嗜酸球浸潤，甚至有出血的肝細胞壞死。但著者未能由電子顯微鏡確認到明顯的病毒粒子。

臺灣省家畜衛生試驗所