Outbreaks of Egg-Drop Syndrome-1976 in Japan and Pathogenicity of Isolated Virus (JPA-1 Strain) for Laying Hens

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In 1976, a syndrome causing depressed egg production associated with the laying of soft-shelled and shell-less eggs was first described in the Netherlands by van Eck et al.,[10] who suggested the possible involvement of fowl adenovirus (FAV) in the syndrome. Later, McFerran et al. isolated several hemaggultinating adenoviruses from affected hens in Northern Ireland[6] and demonstrated a correlation between the syndrome and the isolate.[4,5] Outbreaks of egg drop syndrome-1976 (EDS-76) have been limited to western European countries. The present authors observed falls in egg production similar to EDS-76 in two farms in Japan, diagnosed them as EDS-76 by serological and virological examinations, and reported first in Japan.[12] Then, EDS-76 was produced experimentally in laying hens by the isolated virus.[13]

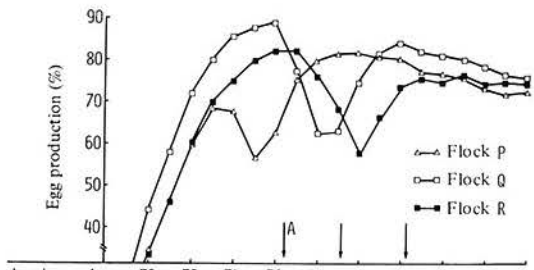
This report deals with diagnosis of EDS-76 in field case,[12] and clinical, virological and pathological examinations in experimental case of EDS-76.[8,13]

Diagnosis of the field case

1) Clinical observations

Conditions similar to EDS-76 occurred in two broiler breeding farms. In both farms, all chickens had been vaccinated against Marek's disease, infectious bronchitis, Newcastle disease, fowl pox, and avian encephalomyelitis before they began to lay.

On a farm (farm I), falls in egg production were recognized from December 1978 to May 1979. Nine out of 14 broiler breeding flocks in the farm were affected, one after another. Production fell suddenly when the flocks were 30 to 55 weeks of age and lasted 3 to 7 weeks. The rates of maximum falls in egg production ranged from 6 to 17% when compared with the predicted production curves for the breed of chicken (Table 1). Depressed egg production was accompanied by the laying of shell-less, soft-shelled, and thin-shelled eggs associated with loss of egg-shell pigment. On the other farm (farm II), located in another prefecture and in which line of broiler breeding hens differed from that in farm I, egg production fell in 5 flocks 32 to 33 weeks of age and lasted 4 to 5 weeks (Table 1). Abnormalities of egg shells were similar to those in farm I. The rates of maximum falls in egg production ranged from 20 to 25% when compared with the



Flock	HI antibody to EDS-76		
P	10/10[B]	9/10	NT[C]
Q	0/10	10/10	10/10
R	0/10	8/9	10/10

Fig. 1. Egg-production graph and development of hemagglutination-inhibition (HI) antibody to EDS-76 virus in three flocks in farm II
A) When sera collected. B) No. sera positive/no. tested. C) Not tested.

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Table 1. Falls in egg production recognized in farms I and II

Farm	Flock	Egg production fall			Maximum fall (%)
		Date	Age (wks)	Duration (wks)	
I	A	Dec. 26, 78	41	6	10
	B	Feb. 6, 79	31	4	16
	C	Feb. 8, 79	49	3	16
	D	Feb. 13, 79	42	5	13
	E	Feb. 28, 79	35	4	10
	F	Mar. 8, 79	55	3	6
	G	Mar. 23, 79	30	6	17
	H	Apr. 12, 79	49	7	10
	I	May. 15, 79	37	4	10
II	N	Aug. 14, 79	32	4	20
	O	Sept. 4, 79	35	4	25
	P	Nov. 6, 79	34	5	23
	Q	Nov. 28, 79	37	5	25
	R	Dec. 4, 79	38	5	22

predicted production curves for this breed of chicken (Fig. 1).

1) Serological observations

Antibody to EDS-76 was surveyed by hemagglutination inhibition (HI) test in the two farms. The HI test was carried out by the conventional microtiter method using 0.025-m*l* volumes. Eight hemagglutinating units of antigen were reacted against 2-fold serial dilutions of test sera beginning at 1/4. The highest serum dilution showing complete inhibition of hemagglutination (HA) pattern was taken as the titer, and the titers higher than 1/4 were regarded as HI antibody positive.

Table 2 shows results of serological tests in farm I. All 57 sera collected after onset of the falls in egg production had HI antibody to EDS-76 virus. The HI antibody titers ranged from 1/16 to 1/512. No sera collected before onset of the falls, however, had HI antibody. The number of sera positive to FAV increased significantly after egg production fell. It is presumed that the seroconversion to FAV was recalled by subsequent infection of EDS-76 virus, as described by McFerran et al.[7] No seroconversion to infectious bronchitis virus was observed. In farm II, developments of HI antibody to EDS-76 virus were also closely related to falls in egg production (Fig. 1). The results of serological observations suggest the disease as EDS-76.

Table 2. Antibodies to egg drop syndrome-1976 (EDS-76) virus, fowl adenovirus (FAV), and infectious bronchitis virus (IBV) in sera collected before and after onset of falls in egg production in 11 flocks of farm I

Fall in egg production	Flock	Antibody to					
		EDS-76[a]		FAV[b]		IBV[c]	
		Before onset	After onset	Before onset	After onset	Before onset	After onset
Yes	A		12/12[d]		9/12		3.8[e]
	B		9/9		5/9		3.1
	C		14/14		8/14		3.9
	G	0/5	11/11	1/5	11/11	3.2	3.5
	I	0/5	11/11	0/5	10/11	3.6	3.4
	E	0/9		2/9		3.4	
	H	0/5		3/5		3.9	
No	J	0/5		1/5		3.4	
	K	0/5		0/5		3.4	
	L	0/5		1/5		3.3	
	M	0/5		2/5		3.4	
Total or mean		0/44	57/57	10/44	43/57	3.45	3.54

a) Hemagglutination-inhibition test
b) Agar-gel precipitation test
c) Neutralization test
d) No. sera positive/no. tested
e) Geometric mean titer of the reciprocal of the serum dilution causing 50% plaque reduction expressed by logarithm based on 10

3) Virus isolation

Chicken kidney (CK) cell culture was used for virus isolation. In farm I, portions of trachea, oviduct, and rectum were harvested from 12 hens selected from 2 affected flocks. In farm II, several organs were collected from three hens in flock R when the fall was about maximum, and 146 cloacal swabs and 49 buffy coats were collected from three affected flocks. The CK cells inoculated with the viral samples were examined daily for evidence of cytopathic effect (CPE) for more than 2 weeks. When the CPE appeared, or when the examination ended, cells were disrupted by freezing and thawing and were centrifuged at 4,000 rpm for 5 min. The supernatant was tested for HA, using 0.8% chicken erythrocytes.

In this trial, 10 hemagglutinating agents were isolated from cloacal swabs and one from an oviduct in farm II. All the hemagglutinating agents isolated produced inclusion bodies and antigens reacted with fluorescent antibody against BC14 strain[2] of EDS-76 virus.

From the results of clinical, serological and virological observations, the disease occurred on the two farms were diagnosed as EDS-76. And they were the first cases of EDS-76 recognized in Japan.

Biological and physicochemical properties of the virus

One of the isolates was cloned by the plaque-picking method on a monolayer of chicken-embryo-liver (CEL) cells; it was named JPA-1 strain.

The JPA-1 strain possessed the same antigenicity as the BC14 strain by agar-gel precipitation, HI, immunofluorescent, and neutralization tests. There was no crossing in any tests between the JPA-1 strain and the TR-59 strain of FAV. So the JPA-1 strain was identified as EDS-76 virus.

The virus was stable against organic solvents and at pH 3. The infectivity of the virus was not affected at 50°C for 60 min, but declined in titer by 2.8 $\log_{10}$ in the presence of 1 M magnesium chloride. Infectious virus showed a decline in titer of 1.9 $\log_{10}$ at 56°C for 60 min and was completely destroyed at 60°C.

The complete viral particles were 70 to 80 nm in diameter in electron microscopy, and 1.33 g/m*l* in CsCl equilibrium density-gradient centrifugation.

The virus grew well in CEL and CK cell cultures as evidenced by infectivity and hemagglutinin production. In CEL cells, intracellular and extracellular virus began to increase logarithmically between 16 and 18 hr postinoculation (PI), reaching maximums {$10^{9.2}$ and $10^{8.5}$ plaque-forming units (PFU)/m*l*, respectively} at 48 hr. Intracellular hemagglutinin began to appear from 18 hr PI, reaching a maximum (10,240 HA units) at 48 hr, whereas extracellular hemagglutinin began to appear from 48 hr. Intranuclear fluorescent antigens in infected cells began to appear from 16 hr PI (Fig. 2).

The results of biological and physicochemical studies confirmed the results of others.[1,3,7,9]

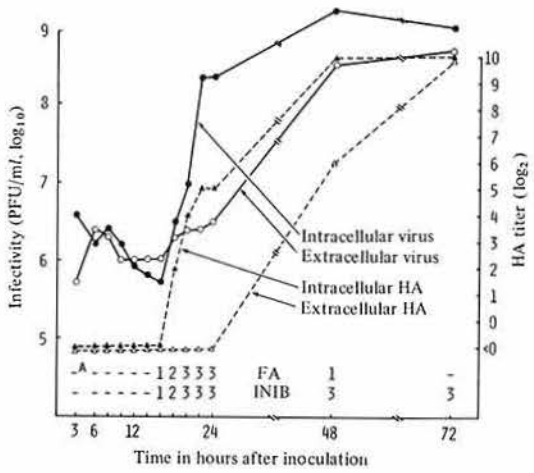

Fig. 2. Growth curves of the virus and hemagglutinin (HA), and appearance time of fluorescent antigen (FA) and intranuclear inclusion body (INIB) of the JPA-1 strain in chick embryo liver cells
A) Degree of FA or INIB:-, negative; 1, mild; 2, moderate; 3, severe.

Experimental reproduction of EDS-76 by the JPA-1 strain

Apparently normal conventional Rhode Island Red laying hens that lacked HI antibody to the

JPA-1 strain were used to investigate the pathogenicity of the virus. Thirty-two 200-day-old hens were divided equally into 2 groups. The hens were caged individually, each group in a separate room, and allowed to acclimate for 12 days. Each hen in one group was inoculated orally with 0.2 m*l* culture medium containing $10^{8.2}$ PFU of the JPA-1 strain at the 4th passage level in CEL cells and observed for 80 days PI. The sixteen hens in the other group were maintained as uninoculated controls and observed for 60 days PI.

Some hens in the inoculated group had diarrhea from 10 days PI but no other clinical signs. Egg production fell from 94% to 50% between 13 and 16 days PI and returned to more than 90% after several days (Fig. 3). However, when the abnormal eggs were excluded from the data, the egg-production rate was 17% between 13 and 16 days PI; it then recovered slowly and reached 67% by the end of the experiment. Nine hens stopped laying for more than 3 days.

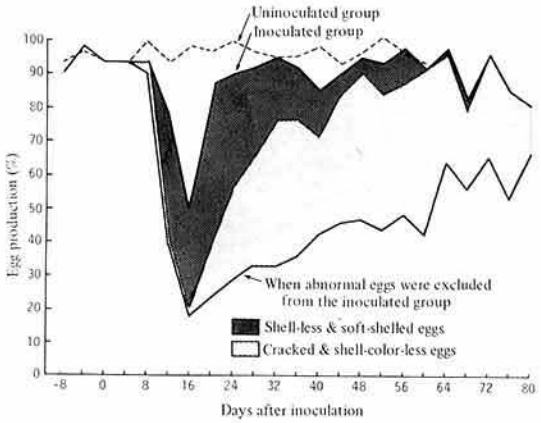

Fig. 3. Egg-production curves of uninoculated hens and hens inoculated with the JPA-1 strain. Mean egg-production rate in each group was calculated every four days.

Abnormal eggs, such as shell-less, soft-shelled and cracked eggs and those with loss of shell pigment (Plate 1), were laid by 15 hens. Eggs with loss of pigmentation were first recognized 8 days PI. Thirteen hens began to lay abnormal eggs between 8 and 11 days PI, and three hens did not begin to lay normal eggs again until 80 days PI. Shell-less or soft-shelled eggs were laid mostly between 17 and 20 days PI; the eggs with loss of pigmentation and cracked eggs increased later (Fig. 3); eggs with loss of pigmentation and cracked eggs had thinner shells than apparently normal eggs. The surface of thin-shelled eggs had a rough, sandpaper-like texture. Misshapen and ridged eggs, however, were not seen. Out of 1,136 eggs laid, 517 (45.5%) were abnormal, and they consisted of 163 shell-less or soft-shelled eggs (14.3%) and 354 eggs with loss of pigmentation and/or cracks (31.2%). The internal qualities, yolk and albumen, were visually normal.

The uninoculated group laid no abnormal eggs. Egg production was more than 94% during the 60 days, and no hen stopped laying for more than 2 days.

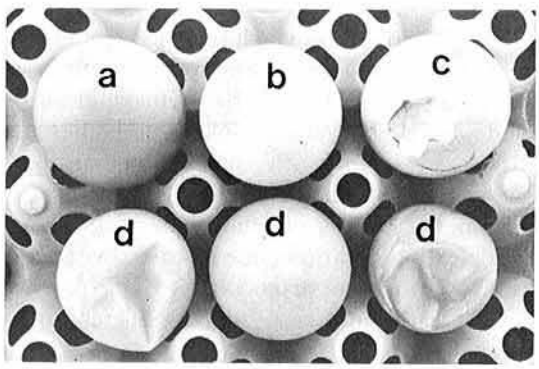

Plate 1. Normal brown egg (a) and abnormal eggs, such as loss of egg-shell pigment (b), cracked (c), and soft-shelled (d) laid 16 days PI

Distribution of the virus in organs

Twenty 275-day-old Rhode Island Red laying hens were used. Each of 16 hens was inoculated orally with 0.2 m*l* culture medium containing $10^{6.1}$ PFU of the JPA-1 strain at the 3rd passage level in CEL. Two hens/each day were killed 1, 3, 5, 7, 10, 14, 21, and 28 days PI. Two uninoculated control hens were killed before inoculation and 2 at the end of the experiment.

The virus was recovered from the various organs examined 3 to 7 days PI, exclusive of the kidneys, ovary, uterus, and serum. But the virus

Table 3. Distribution of the virus and fluorescent antigens in organs of hens inoculated orally with the JPA-1 strain

	Inoculated																Uninoculated			
Days PI:	1		3		5		7		10		14		21		28		0		28	
Hen No.:	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	1	2	19	20
Liver	—[a]	—	—	—	—	3.1[b]	—	3.0	—	—	—	—	—	—	—	—	—	—	NE[c]	NE
Spleen	—	—	—	0.7	—	4.2	—	0.4	—	—	—	—	—	—	—	—	—	—	NE	NE
Pancreas	—	—	—	—	—	3.2	—	1.9	—	—	—	—	—	—	—	—	—	—	NE	NE
Kidneys	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	NE	NE
Trachea	—	—	—	—	—	3.1	1.4	1.4	—	—	—	—	—	—	—	—	—	—	NE	NE
Lungs	—	—	—	—	—	1.4	—	—	—	—	—	—	—	—	—	—	—	—	NE	NE
Jejunum	—	—	—	—	—	2.8	—	—	—	—	—	—	—	—	—	—	—	—	NE	NE
Ceacal tonsil	—	—	—	—	—	2.9	1.1	—	—	—	—	—	—	—	—	—	—	—	NE	NE
Rectum	—	—	—	—	—	3.2	2.7	—	—	—	—	—	—	—	—	—	—	—	—	—
Ovary	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	NE	NE
Oviduct																				
Magnum	—	—	—	—	—	2.6	—	2.6	—	—	—	—	—	—	—	—	—	—	—	—
Isthmus	—	—	—	—	—	—	—	2.4	—	—	—[d]	—	—	—	—	—	—	—	—	—
Uterus	—	—	—	—	—	—	—	—	—	—[d]	—[d]	—[d]	—	—	—	—	—	—	—	—
Serum	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	NE	NE
Antibody[e]	<2	<2	<2	<2	<2	<2	3	<2	8	8	7	10	10	5	9	8	<2	<2	<2	<2

[a] Negative in 10% suspensions of the organ
[b] Log-PFU per m*l* of 10% suspensions of the organ
[c] Not examined
[d] Fluorescent antigens were observed in a section of the organ.
[e] The figure indicates the titer of hemagglutination-inhibition antibody expressed by logarithm based on 2.

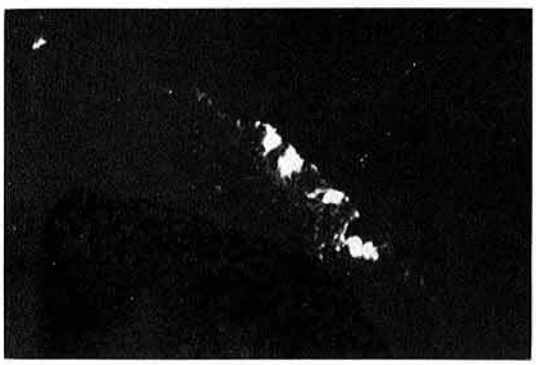

Plate 2. Fluorescent antigens in the epithelial cells of the uterus observed 14 days PI

did not grow well and the titers were less than $10^{4.2}$ PFU per m*l* of 10% suspension (Table 3).

No fluorescent antigen was detected before 7 days PI, but it was detected in uterus and isthmus 10 and 14 days PI (Table 3). The antigens were detected a lot in the epithelial cells and desquamated cells from the epithelium of the uterus (Plate 2), whereas detected a little in the epithelial cells of the isthmus.

Pathological changes

1) Macroscopic findings

The same hens as used for the examination of virus distribution were used for pathological examination.

Mild splenomegaly with an enlargement of white pulp was noted in hens 5 and 7 days PI. The uterus became slack with edema in the subserosa 14 days PI. The mucosal folds were swollen edematously and covered with a whitish opaque exudate in one hen (Plate 3). In the other hen on day 14, a yellow chalky exudate was found among the uterine mucosal folds. Soft atrophic ovarian follicles were observed in the ovaries of one bird each 14 and 21 days PI.

2) Microscopic findings

(1) Oviduct

Histological changes were generally noted in the oviduct and especially in the uterus (Tables 4 and 5). In severely affected uterus on day 14,

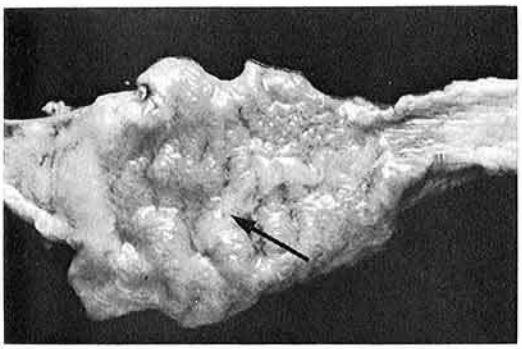

Plate 3. Uterus at 14 days PI
Uterine folds are edematous and swollen. Whitish mucous exudate is on the surface of mucosa (arrow).

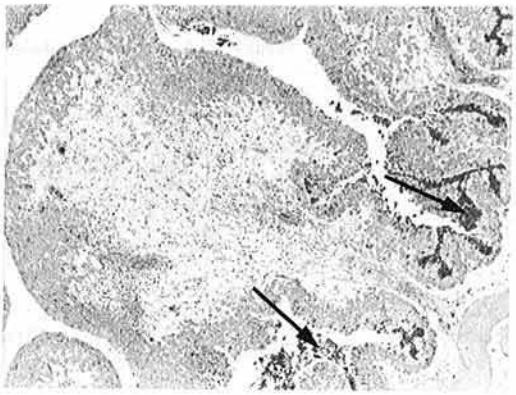

Plate 4. Uterus at 14 days PI
Uterine fold (F) dilated remarkably due to edema and cell infiltration in lamina propria and submucosa. Arrows indicate exudate in lumen. HE staining, ×33

the mucosal folds were distended extensively with cell infiltration and edema, and the surface of the epithelium appeared uneven (Plate 4). Much exudate was noted in the cavity. Secretory granules and cilia decreased or lost in most epithelial cells which contained intranuclear inclusion bodies (Plate 5). Most intranuclear inclusion bodies were stained homogeneously pale or dark with hematoxylin and some contained a few granules stained with both hematoxylin and eosin. In this stage the uterine glands were mainly composed of atrophic cells arranged in a rosette or tubular form. In hens on day 21 and

Table 4. Incidence of histological lesions in laying hens inoculated with the JPA-1 strain

Hen No.	Days PI	Ovary[a]	Lymphocyte infiltration						
			Oviduct					Peritoneum	Liver
			Inf.	Mag.	Isth.	Ute.	Vag.		
1	0-cont.	−	−	−	−	−	−	−	−
2	0-cont.	−	−	−	−	−	−	−	−
3	1	−	−	−	−	−	−	−	−
4	1	−	−	−	−	−	−	−	−
5	3	−	−	−	−	−	−	−	−
6	3	−	−	−	−	−	−	−	−
7	5	−	−	−	−	−	−	−	+
8	5	−	++	−	−	−	−	−	−
9	7	−	++	−	−	−	−	−	−
10	7	−	+	−	−	+	+	−	−
11	10	−	++	−	+	−	+	+	−
12	10	−	+	−	−	+B	−	+	+
13	14	+	+F	+	+B	+++B	++B	+++F	−
14	14	+	++F	−	+	+++B	++	++	−
15	21	+	+F	−	+	++F	−	+F	+F
16	21	−	+	−	−	+F	+	−	−
17	28	−	+F	−	+F	+F	+F	+F	+
18	28	−	+F	−	+	+F	+F	+F	−
19	28-cont.	−	−	−	−	−	−	−	−
20	28-cont.	−	−	−	−	−	−	−	−

[a] Degeneration of ovarian follicles
Cont.: Uninoculated control hens, Inf.: Infundibulum, Mag.: Magnum, Isth.: Isthmus, Ute.: Uterus, Vag.: Vagina
−: No, +: Mild, ++: Moderate, and +++: Severe changes
B: Intranuclear inclusion body, F: Lymphoid follicle

Table 5. Histopathological changes in affected uterus of hens inoculated with the JPA-1 strain

Days PI:	7	10	14		21		28	
Hen No.:	10	12	13	14	15	16	17	18
Exudate in lumen	−	−	++	++	−	−	−	−
Epithelial cells								
Inclusion body	−	+	+++	+	−	−	−	−
Degeneration & desquamation	−	+	+++	+++	−	−	−	−
Squamous cells	−	−	+	+++	−	−	−	−
Atrophy of glands	−	−	+++	+++	+	−	−	−
Edema of folds	−	+	+++	++	+	−	−	−
Cell infiltration in folds								
Lymphocytes	+	+	+++	+++	++	+	−	+
Heterophils	−	+	++	+	−	−	−	−
Plasmacytes	−	−	+	++	+	+	+	+
Lymphoid follicles	−	−	−	−	++	+	++	+
Vasculitis	−	−	+	++	−	−	−	−
Muscular layer								
Cell infiltration	−	−	++	+	+	−	−	−
Edema	−	−	++	+	−	−	−	−

−: No, +: Mild, ++: Moderate, and +++: Severe changes

28, mild infiltration with lymphocytes and plasmacytes was discerned and lymphoid follicles were formed around blood vessels in the uterine folds (Plate 6).

The infundibulum was affected with lymphocytic infiltration after 5 days PI. Intranuclear inclusion bodies were seen in the mucosal epithelium of the isthmus and vaginal gland region in a hen 14 days PI.

(2) Other organs

Changes in other organs were rather mild. In the ovary, some mature ovarian follicles revealed degeneration, desquamation and invaginative growth of granulosa cells in 3 hens 14 and 21 days PI. Ova of these follicles were degenerative and stained unevenly.

In the spleen, mild swelling and proliferation of reticular cells around the sheathed arteries 3 and 5 days PI.

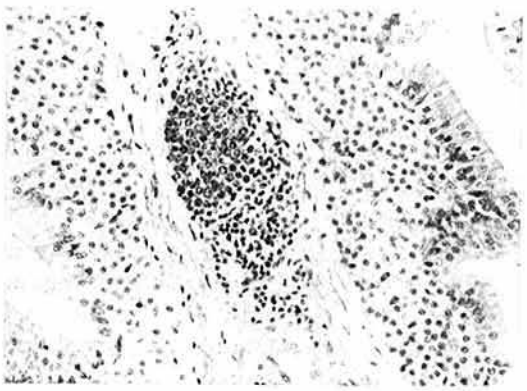

Plate 6. A lymphoid follicle formed in a fold of the uterus 28 days PI
HE staining, ×250

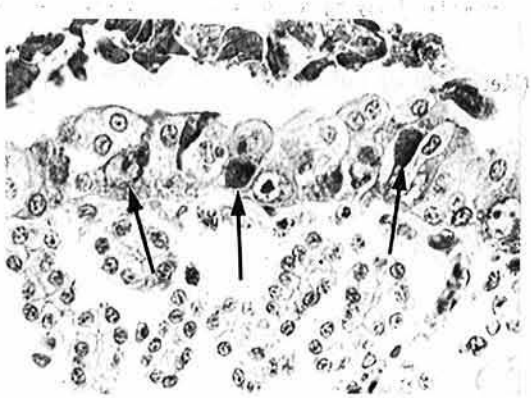

Plate 5. Intranuclear inclusion bodies (arrows) observed in the epithelial cells of uterus 14 days PI
HE staining, ×500

3) Electron microscopic findings

Immediately after heart bleeding small pieces were obtained from middle part of the magnum, isthmus and uterus.

The nuclei of epithelial cells containing inclusion bodies were irregular in shape, and various amounts of virus particles and electron-dense

amorphous substance were seen in them. Virus particles were also recognized in both nucleus and cytoplasm of desquamated, degenerative epithelial cells in the uterine cavity. Macrophages, infiltrating in and beneath the epithelial layer, contained virus particles and degenerative microorganelles.

Conclusions

The falls in egg production which occurred in the two farms were diagnosed as EDS-76 by clinical and serological observations and the virus isolation. They were the first cases of EDS-76 recognized in Japan.

By the experimental inoculation of the laying hen with the JPA-1 strain, clinical signs, abnormality of the eggs, target organs of the virus and pathological changes became clear. Main clinical sign was a diarrhea and it was detected from 10 days PI. Inoculated hens laid eggs with abnormal shell from 8 days PI and it became severe during 13 and 16 days PI. The virus grew mainly in the epithelial cells of uterus as evidenced by detection of large amount of fluorescent antigen, intranuclear inclusion bodies and viral particles. Moreover histological changes such as degeneration and desquamation of the epithelial cells, edema of the fold and atrophy of the shell-grand were detected in the uterus.

According to these findings, it is suspected that the virus exerts a direct effect on the uterus, then the uterus is suffered from forming the eggs with complete shell.

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