Isolation of the Virus of Egg Drop Syndrome 1976 (EDS-76) in a Japanese Outbreak

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ABSTRACT. We observed outbreaks of an egg drop syndrome similar to EDS-76 over the period from October, 1979 to March, 1980. A hemagglutinating virus (H-162) was isolated from the faces of a hen in a broiler breeding flock and shown to be serologically identical to the EDS-76 virus by the double immunodiffusion and hemagglutination-inhibition (HI) tests. The appearance of HI antibody to this virus was associated with the syndrome. The replication of the H-162 virus was inhibited with 5-iodo-2'-deoxyuridine and its infectivity was resistant to treatment by ethyl ether or chloroform. The virus was stable at pH 3.7 and readily filtered through membrane filters with 100 nm or greater pore sizes, but not through 50-nm filters. The virus multiplied with cytopathic effect in cultures of chick embryo liver and kidney cells and fibroblasts and chicken kidney cells. Eosinophilic inclusion bodies were observed in the nuclei of infected cells. Electron microscopy revealed typical adenovirus particles often aggregated in paracrystalline array forming a cubic lattice in the nuclei of thin-sectioned cells. The virus multiplied to high titers in the allantoic cavity of duck embryos, whereas no viral growth was shown in chicken embryos. The virus agglutinated chicken erythrocytes and the reaction was specifically inhibited with antiserum against the virus.

In 1976, a new syndrome characterized by depressed egg production and laying soft-shelled and shell-less eggs was first described in the Netherlands by Van Eck *et al.* [7], who attributed the syndrome to an adenovirus infection on serological grounds. This condition has since become known as "Egg Drop Syndrome 1976" (EDS-76). Shortly after this observation, McFerran *et al.* [5] reported the isolation of an adenovirus (strain 127) from such affected flocks in Northern Ireland and noted that the appearance of antibody to this virus was associated with the syndrome. Adenovirus strains serologically identical to the 127 strain have also been isolated in other European countries [2, 6]. Recently this virus was isolated from flocks with egg drop syndrome in Japan [8] and in

Australia [3].

Over the period from October, 1979 to March, 1980, we observed outbreaks of illness very similar to EDS-76 in broiler and layer breeding flocks in Okayama Prefecture, Japan. We isolated a virus which was identified as the EDS-76 virus. This paper describes the virus isolation, the properties of the virus and serological evidence for its etiological role.

MATERIALS AND METHODS

Cell cultures: Chick embryo liver (CEL) cells were obtained from 14-day-old embryos. Cell monolayers were prepared in 6-cm plastic dishes, in semi-microplates (4×6 wells) and in microplates (8×12 wells) by seeding 5, 1 and 0.1 ml, respectively, of a trypsinized cell suspension of 1×10^6 cells/ml, and incubating them at

37°C for 4 to 5 days in Eagle's minimum essential medium (MEM) containing 10% fetal calf serum. The maintenance medium was MEM containing 0.2% fetal calf serum. Chick embryo kidney (CEK) cells were obtained by trypsinization of 18-day-old embryos. Cell packs were diluted 300-fold, delivered in 1-ml volumes in wells of semi-microplates or in 2-ml volumes in 3-cm dishes, and incubated at 37°C for 2 to 3 days. The growth medium was MEM containing 5% calf serum and 5% tryptose phosphate broth (TPB) and the maintenance medium was MEM containing 0.5% calf serum and 2% TPB. Chick embryo fibroblasts (CEF) were prepared from 11-day-old embryos. The trypsinized cell suspension, 1×10^6 cells/ml, in MEM containing 10% calf serum was seeded in 1-ml volumes in wells of semi-microplates and incubated at 37°C for 2 days. The maintenance medium was MEM containing 1% calf serum. The chick embryos used were all obtained from a specific pathogen-free flock (Spafas Inc., U.S.A.). Cultures of chick kidney (CK) cells were prepared from 1 to 3-week-old specific pathogen-free chickens in the same manner as those of CEK cells. HeLa and Vero cells were grown in MEM containing 10% calf serum and maintained in MEM containing 2% calf serum. HEL R-66 cells were grown in MEM containing 2% calf serum and the serum content of the maintenance medium was reduced to 0.2%.

Viruses: The BC-14 strain [2] and the JPA-1 strain [8] of the EDS-76 virus were kidney supplied by Dr. S. Yamaguchi, Poultry Disease Laboratory, National Institute of Animal Health, Japan. The strains were passaged twice in CEL cell cultures before used in this study. The Ote strain of fowl adenovirus type 1 [4] was kidney supplied by Dr. H. Kawa-

mura, Poultry Laboratory, National Institute of Animal Health, Japan. The strain was passaged 3 times in CK cell cultures before used in this study.

Infectivity assay: The new isolate, H-162, and BC-14 and JPA-1 strains were titrated in CEL cell cultures prepared in microplates. Serial decimal dilutions of the test material were each inoculated in 0.1-ml volumes into 5 wells and incubated at 37°C. After incubation for 5 days, the culture fluid was examined for hemagglutinating activity with 0.5% chicken erythrocytes, and then the TCID₅₀ titer was calculated. The Ote strain was assayed in CK cell cultures prepared in semi-microplates by inoculating each of serial decimal dilutions of the test material in 0.1-ml volumes into three wells. After virus adsorption at 37°C for 60 min, each well was fed with 1 ml of maintenance medium. After incubation for 5 days at 37°C, the cultures were examined for cytopathic effect and the TCID₅₀ titer was calculated.

Electron microscopy: Monolayer cultures of CEL cells prepared in 6-cm dishes were infected with $10^{6.5}$ TCID₅₀ of virus and incubated at 37°C after virus adsorption at 37°C for 60 min. After incubation for 24, 48 or 72 hr, the cell sheet was washed once with phosphate buffered saline (PBS; 0.15 M NaCl, 0.01 M phosphate buffer, pH 7.4) and treated with 0.05% trypsin and 0.5 mg/ml EDTA. The dispersed cells were washed twice with PBS containing Mg⁺⁺ and Ca⁺⁺ ions, fixed with 5% glutaraldehyde in 0.05 M cacodylate buffer. After postfixation with 1% osmium tetroxide in 0.1 M cacodylate buffer, the cell specimens were dehydrated in a graded series of ethyl alcohol and embedded in Epon 812. Ultrathin sections were double-stained with a mixture of uranyl acetate and lead and examined under a Hitachi

HU-12 electron microscope.

Hemagglutination (HA) and hemagglutination-inhibition (HI) tests: The HA and HI tests were carried out by the conventional microtiter method. In HA tests, serial twofold dilutions of a virus material were made in 0.05-ml volumes and each dilution was mixed with an equal volume of 0.5% chicken erythrocyte suspension. The diluent used was PBS containing 0.1% gelatin. The tests were read after incubation at room temperature for 30 min. The reciprocal of the highest virus dilution showing partial HA was taken as the titer (HAU/0.05 ml).

For HI tests, the sera were inactivated at 56°C for 30 min and serial twofold dilutions were made in 0.025-ml volumes with the diluent described above. Each serum dilution was mixed with 0.025 ml of diluent containing 4 HAU of antigen and incubated at room temperature for 30 min. The serum-antigen mixtures received 0.05 ml of a 0.5% erythrocyte suspension and were incubated at room temperature for 30 min. The HI titer was expressed as the reciprocal of the highest serum dilution showing complete HI.

The antisera used in HI tests were prepared in 12-week-old specific pathogen-free hens. The hens were inoculated by the oral route with $10^{6.7}$ TCID₅₀ of the BC-14 strain, $10^{7.4}$ TCID₅₀ of the JPA-1 strain or $10^{8.5}$ TCID₅₀ of the Ote strain. Serum was obtained from five hens 21 days post-infection and pooled. The antiserum against the new isolate, H-162, was prepared in 60-day-old specific pathogen-free hens. Seven hens were inoculated by the oral route with $10^{6.9}$ TCID₅₀. Serum was obtained 14 days later and pooled.

Double immunodiffusion: The antigen for the test was prepared by infecting CEL cells with the H-162 virus and har-

vesting when the cytopathic effect was maximal. The antigen of the Ote strain was prepared in CK cells. After three cycles of freezing and thawing and low speed centrifugation to remove gross cell debris, the viral protein was precipitated from the supernatant fluid at 4°C overnight by the addition of an equal volume of a saturated ammonium sulfate solution. The precipitate was suspended in 0.01 volume of distilled water and dialyzed against PBS. The antigen was standardized against the antiserum.

Five milliliters of molten agar (1% Noble agar in PBS, pH 7.4 containing 1% sodium azide) was poured on a slide glass and allowed to harden. Wells, 5 mm in diameter, were punched in the agar. The central and peripheral wells received antigens and serum dilutions, respectively, in 0.05-ml volumes, and the slides were incubated at 37°C for 72 hr.

The antiserum against the new isolate, H-162, used in double immunodiffusion tests was prepared in 10-week-old specific pathogen-free hens. The birds received an intravenous dose of $5 \times 10^{7.5}$ TCID₅₀ and serum was withdrawn 14 days later and pooled. The antiserum against the BC-14 strain, a gift from Dr. H. Kawamura, was obtained from experimentally infected birds.

RESULTS

Virus isolation

Outbreaks of egg drop syndrome were observed in 16 broiler and layer breeding flocks on four farms. The syndrome was quite reminiscent of the EDS-76. Affected birds remained healthy or showed only transient dullness and diarrhea. The decline of egg production began mostly at 30 weeks of age and continued for 3 to 8 weeks. The maximum drop ranged from 5 to 55% of the predicted production rate for the breed of chicken.

Fig. 1. Egg production in a broiler breeding flock (PS).

Egg abnormalities observed were lack of shell pigment, decreased size and shell thickness and soft-shelled or shell-less eggs.

Virus isolation was attempted from fecal specimens collected from three affected flocks at various stages of the outbreak and a virus strain was recovered from a specimen obtained from a broiler breeding flock (parent stock; PS) 13 days after the onset of egg drop. The egg production curve for the PS flock is given in Fig. 1, together with the stage at which the virus was isolated.

The fecal specimen was homogenized to make a 10% suspension in PBS, which was centrifuged at $3,000\times g$ for 10 min. The supernatant fluid was mixed with an equal volume of chloroform and the mixture allowed to stand overnight at 4°C . The water phase separated by centrifugation was inoculated in 0.2-ml volumes into three CEL cell cultures prepared in 6-cm dishes. After virus adsorption at 37°C for 60 min, the inoculated cultures were fed with 5 ml of maintenance medium and incubated at 37°C . None of the inoculated cultures showed cytopathic effect 5 days post-infection. Conse-

quently, a blind passage was made into three CEL cell cultures inoculated with the pooled supernatant fluid after disruption of the cultures by freezing and thawing. The second passage cultures began to exhibit cytopathic effect on the third day. The culture fluid agglutinated chicken erythrocytes. After an additional passage, the isolate was purified by three successive passages at limiting dilution and designated as H-162 strain.

Serological identification

In double immunodiffusion tests, the H-162 strain showed no difference in antigenicity from the BC-14 strain of EDS-76 virus, whereas it showed no crossing with the Ote strain of fowl adenovirus type 1. Cross HI tests indicated that the H-162 strain was serologically identical to the EDS-76 virus, whereas it was quite distinct from the Ote strain (Table 1).

Physicochemical properties

As shown in Table 2, the replication of the H-162 strain was inhibited with 5-iodo-2'-deoxyuridine (IUDR). In this experiment, serial decimal dilutions of a stock virus were each inoculated in 0.1-ml volumes in CEL cell cultures prepar-

Table 1. Cross hemagglutination-inhibition (HI) tests of the H-162 strain with the BC-14 and JPA-1 strains of Egg drop syndrome 1976 virus

Antiserum	HI titer to virus strain		
	H-162	BC-14	JPA-1
H-162	512	512	256
BC-14	128	128	64
JPA-1	256	256	128

Table 2. Effect of 5-iodo-2'-deoxyuridine (IUDR) on replication of the H-162 strain

IUDR (100 µg/ml)	Thymidine (100 µg/ml)	Infectivity (log ₁₀ TCID ₅₀ /ml)
+	—	≤2.5
+	+	7.5
—	+	7.2
—	—	7.5

Table 3. Effect of chloroform, ether, low pH and heating on infectivity and HA activity of the H-162 strain

Treatment	Infectivity	HA activity
Chloroform, 10%, 15 min.	7.5*	2,560**
Ethyl ether, 25%, 18 hr.	7.2	2,560
pH 3.7, 180 min.	6.7	1,280
56°C, 30 min.	2.2	1,024
None	7.5	2,560

* Log₁₀ TCID₅₀/ml; ** HAU/0.05 ml

ed in three wells in semi-microplates. After virus adsorption at 37°C for 60 min, each well was fed with 1 ml of maintenance medium containing 100 µg/ml IUDR. After incubation at 37°C for 5 days, the culture fluid was tested for HA and the TCID₅₀ titer was calculated. Inoculated cultures incubated with 100 µg/ml thymidine or with 100 µg/ml IUDR and 100 µg/ml thymidine were included in the test. Inoculated cultures fed with plain maintenance medium served as the control. As shown in Table 2, strong inhibition of viral growth was shown in the presence of IUDR and this inhibition was countered with thymidine.

The infectivity of the H-162 strain was resistant to chloroform and ethyl ether as shown in Table 3. For the treatment with chloroform, 1.8 ml of a virus suspension was mixed with 0.2 ml of chloroform, and the mixture allowed to stand at room temperature for 15 min after vigorous shaking. The water phase separated by centrifugation was assayed for infectivity. For the ether treatment a mixture of 3 ml of virus suspension and 1 ml of anesthetic ether was incubated at 4°C for 18 hr. The mixture was then centrifuged and the water phase was allowed to stand at room temperature for 4 hr before infectivity assay.

The H-162 strain was stable at pH 3.7 (Table 3). In this test, serial decimal dilutions of a stock virus were made with acidic MEM (pH 3.7) before addition of sodium bicarbonate and they were allowed to stand at room temperature for 3 hr. After adjustment of the pH to neutrality, each dilution was tested for infectivity.

The virus was labile at 56°C (Table 3). The HA titer was not affected by the treatment with ether, chloroform, acidic pH or heating at 56°C (Table 3).

The H-162 strain was readily filtered through membrane filters with 100 nm or greater pore size, but not through 50-nm filters.

Biological properties

The H-162 strain multiplied with cytopathic effect in not only CEL, but also CEK, CEF and CK cell cultures. CEL cell cultures gave the highest viral yield. The cytopathic effect was characterized by the appearance of refractile round cells followed by their enlargement and eventual detachment from the cell sheets (Fig. 2). Eosinophilic inclusion bodies were observed in the nuclei of swollen cells in infected cultures fixed with Bouin's solution and stained hematoxylin and eosin or May-Grünwald-Giemsa stain (Fig. 2).

Electron microscopy of infected cells revealed numerous virus particles in the nuclei, about 80 nm in diameter, resembling adenovirus virions. The particles were often aggregated in paracrystalline array forming a cubic lattice (Fig. 3). No replication of the H-162 strain was shown in mammalian cells such as Vero, HeLa and HEL R-66. The virus replicated to high titers in the allantoic cavity of 14-day-old duck embryos. In an experiment, the allantoic fluid had HA titers as high as 8,000–16,000 five days after inoculation with $10^{4.1}$ TCID₅₀. In contrast, no growth was shown in the allantoic cavity of 10-day-old chicken embryos even though four blind passages were made.

The H-162 strain agglutinated chicken erythrocytes. The reaction was specifically inhibited with antiserum against the virus.

HI antibody in affected flock

HI tests were made on random serum samples obtained from 10 hens 13 days after the onset of egg drop and 40 days later from 20 hens in the PS flock from which the H-162 strain was isolated. As shown in Table 4, much higher antibody titers were obtained with later samples than early ones.

Table 4. Hemagglutination-inhibition (HI) antibody response of the PS flock to the H-162 strain

	Days after the onset	
	13	53
160	512	512
160	512	512
80	512	512
80	256	256
80	256	128
40	128	128
40	128	128
20	128	64
10	64	<2
<5	<2	<2

DISCUSSION

In the present study, we isolated a virus strain (H-162) from the feces of a hen in a broiler breeding flock showing an egg drop syndrome. The syndrome was quite reminiscent of the egg drop syndrome 1976 (EDS-76). The strain was shown to be serologically identical to the BC-14 [2] and JAP-1 [8] strains of EDS-76 virus by the double immunodiffusion and HI tests. We further noted that the appearance of HI antibody to this virus was associated with the syndrome. The presence of this virus in Japanese chickens has recently been reported by Yamaguchi *et al.* [8].

The replication of the new isolate, H-162, was inhibited by the incorporation of IUDR in the culture medium, as reported with EDS-76 virus [1, 8], indicating that the isolate is a DNA virus. The infectivity of the H-162 strain was resistant to treatment with either ethyl ether or chloroform coinciding with the results with EDS-76 virus [1, 8]. The H-162 strain was stable in acidity as was EDS-76 virus [1, 8]. The virus was labile at 56°C and readily filtered through membrane filters with 100 nm or greater pore sizes, but not through 50-nm filters.

The H-162 strain multiplied with cytopathic effect in cultures of chick embryonic liver and kidney cells and fibroblasts and chicken kidney cells as reported with EDS-76 virus [1, 5]. Eosinophilic inclusion bodies were observed in the nuclei of infected cells. Electron microscopy revealed typical adenovirus particles often aggregated in paracrystalline array forming a cubic lattice in the nuclei of thin-sectioned infected cell. Electron microscopic observations of cultured cells infected with EDS-76 virus has been reported by Adair *et al.* [1]. No replication of the H-162 strain was shown in

mammalian cells such as HeLa, Vero and HEL R-66.

The virus replicated to high titers in the allantoic cavity of duck embryos, whereas no viral growth was shown in chicken embryos. Firth *et al.* [3] has also reported yield of EDS-76 virus of high titer after inoculation into duck embryos.

The H-162 strain agglutinated chicken erythrocytes as reported with EDS-76 virus [1, 5]. The etiological role of the isolated virus was confirmed by the finding that much higher HI titers were obtained with serum samples collected at a late stage of the outbreak than those collected earlier from the affected flock from which the H-162 strain was isolated.

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EXPLANATION OF FIGURES

Fig. 2. Cytopathic effect of the H-162 strain in chick embryo liver cell cultures: (1) Unstained control cells, (2) 48 hr after virus infection, unstained, (3) Control cells, hematoxylin and eosin staining, and (4) 48 hr after virus infection, hematoxylin and eosin staining.

Fig. 3. Electron micrographs of chick embryo liver cells infected with the H-162 strain 48 hr earlier: (1) A nucleus containing numerous virus particles, and (2) Virus particles aggregated in paracrystalline array.

要 約

産卵低下症候群 1976 (EDS-76) ウイルスの分離: 東原 稔・高井伸二・日高温子・宝達 勉, 蛭間 正巳・渡辺義計¹⁾・松本 稔²⁾ (北里研究所付属家畜衛生研究所, ¹⁾林薬品家畜衛生研究所, ²⁾北里研究所)——1979年10月から1980年3月にかけて発生した, EDS-76 によく似た産卵異常を主徴とする疾病の病原検索を行なったところ, ブロイラー種鶏群の糞便からウイルス(H-162株)が分離された。H-162株は二重免疫拡散法, 血球凝集抑制(HI)試験により既知の EDS-76 ウイルスと区別できなかった。罹患鶏群ではこのウイルスに対する抗体が産生された。H-162株の増殖は 5-iodo-2'-deoxyuridine により阻止され, その感染性はエーテル, クロロフォルムならびに酸処理(pH 3.7)に抵抗性であった。H-162株は平均孔径 100 nm 以上のメンブランフィルターで容易に透過されたが, 50 nm のフィルターは通過しなかった。ニワトリ胎児の肝, 腎ならびに線維芽細胞, ニワトリ腎細胞などの培養で細胞変性をともなって増殖した。H-162株感染細胞の核内にはエオジン好性の封入体が観察された。感染細胞の電顕観察では核内に定型的なアデノウイルス粒子が認められ, それらはしばしば結晶状に配列していた。H-162株は14日齢发育アヒル卵の尿膜腔内でよく増殖したが, 10日齢发育鶏卵の尿膜腔内では増殖しなかった。

Fig. 2

Fig. 3