Studies on Equine Adenovirus

I. Characteristics of an Adenovirus Isolated from a Thoroughbred Colt with Pneumonia

Shin-ichiro KONISHI, Ryô HARASAWA, Masami MOCHIZUKI,
Hiroomi AKASHI and Manabu OGATA

*Department of Veterinary Microbiology, Faculty of Agriculture,
University of Tokyo, Bunkyo-ku, Tokyo 113*

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Abstract. Two cytopathogenic agents were isolated in horse kidney cell cultures from the bronchus and lung of a three-month-old Thoroughbred colt with pneumonia presumed to be caused by bacterial infection. They were propagated in equine fetal dermis cells with cytopathogenic effect and development of intranuclear inclusions similar to those in primary horse kidney cell cultures. The isolate from the pneumonic lesion was used as a representative for characterization, because both isolates were identical in the cross serum neutralization (SN) test.

The isolate was found to contain DNA because of its replication inhibited by 5-iodo-2'-deoxyuridine. Its buoyant density was 1.29 g/cm³. The virion had a cubic symmetry without envelope, and measured approximately 74 nm in diameter. The virus was resistant to chloroform and ether. It was slightly inactivated at pH 3.0 for 3 hours. Hemagglutination was observed in human (type O), guinea pig, horse and rat erythrocytes. The equine isolate shared a common complement-fixing antigen with a canine adenovirus, but it did not react with this virus in the SN test. A serologic survey on a stud from which the foal in the present study was derived demonstrated a prevalence of adenoviral infection.

Adenoviruses have been isolated from foals with respiratory disease in U.S.A. [2, 5, 12, 17-19] and Canada [10], and from clinically normal foals in Australia [9, 20] and Germany [15], but not from adult horses as yet. Most reports have concerned the virus isolation only from Arabian foals with respiratory infections. This paper first describes the isolation, characterization and serological properties of an adenovirus from pneumonic lung tissue of a Thoroughbred colt in Japan.

Materials and Methods

Clinical history of the foal: On April 6, 1972, a

seven-week-old Thoroughbred colt on a stud in Ibaraki Prefecture developed a temperature of 39.7°C and mild rales with coughing in addition to a seromucoid nasal discharge. Antibiotics were administered continually to the foal. Nevertheless, the temperature remained a range from 39.0 to 40.5°C and the dyspnea did not disappear. On May 14, the animal died and was necropsied. Autopsy revealed purulent pneumonia responsible for bacterial infection. Specimens from the liver, kidney, bronchus and pneumonic lung tissue were subjected to microbiological examination. *Corynebacterium* sp. was isolated massively from the pneumonic lung tissue and suspected to be a cause of death. Viral agents were also isolated from bronchus and pneumonic lung tissue.

Cell cultures and medium: An equine fetal dermis (EFD) cell line was grown in Eagle's minimal

ウマのアデノウイルスに関する研究 I. サラブレッド仔ウマの肺炎から分離したアデノウイルスについて:
小西信一郎・原澤 亮・望月雅美・明石博臣・尾形 学 (東京大学農学部家畜微生物学教室)

essential medium (MEM) supplemented with 10% fetal calf serum (FCS). It was maintained in MEM supplemented with 2% FCS. The other cell cultures used in the present study were grown by the method previously reported [11].

Viruses: The viral isolate used for characterization was originated from the pneumonic lung at the eighth to twelfth passage level in cell cultures. The Bucyrus strain of equine arteritis virus (EAV) and the D43 strain of infectious canine hepatitis (ICH) virus [14] were employed as controls.

Titration of virus: The viral titrations of equine isolate and EVA were conducted in EFD cell monolayer, and that of ICH virus was in primary dog kidney cell culture. Fifty per cent end point titers of tissue culture infectious doses ($TCID_{50}$) were calculated by the Reed and Muench formula [16].

Electron microscopy: Thin sections prepared from the infected EFD cells were stained with uranyl acetate and lead nitrate. For negative staining, viral cultures in Roux bottles were subjected to 3 freeze-thaw cycles, and clarified by low centrifugation. The supernatant fluid was concentrated 1:100 by ultrafiltration, then diluted 1:1 with 2% phosphotungstic acid at pH 7.0, and placed onto collodion-coated and carbon-stabilized grids for examination with a Hitachi HU-12 electron microscope at 75 kv.

Nucleic acid type: The nucleic acid type of the isolate was assumed by 5-iodo-2'-deoxyuridine (IUDR). The EFD cell monolayers were inoculated with 300 $TCID_{50}$ /0.2 ml of test and control viruses. Maintenance medium containing $10^{-4.0}$ M IUDR was added to the cultures, but the controls received maintenance medium only. The cultures were harvested every day for titration.

Sensitivity to chloroform and ether: The procedures used to determine sensitivity to chloroform and ether treatments were those described by Feldman and Wang [7] and by Andrewes and Horstman [1], respectively.

pH sensitivity: The isolate and control virus were diluted 1:10 in McIlvaine buffer at pH levels from 3.0 to 7.0. Viruses in the same dilution in MEM were included as controls. After being allowed to stand at room temperature for 0.5 or 3 hours, the mixture was assayed for infectivity.

Hemagglutinating activity: Test erythrocytes were collected from the following animal species: human (type O), horse, rat, guinea pig, fowl, sheep, pig, dog, cat, hamster and mouse. Hemagglutination was examined in Dulbecco's 0.01 M phosphate-buffered saline (PBS) at 4 and 37°C.

Buoyant density: Culture of the equine isolate was centrifuged at 3,000 rpm for 10 minutes. The

supernatant was further centrifuged at 100,000 g for 2 hours. The pellets were suspended in PBS, layered onto a preformed CsCl density gradient, and centrifuged at 100,000 g for 24 hours at 4°C. Ten fractions were collected and examined for density and hemagglutinability. Then they were diluted 1:100 in maintenance medium and assayed for infectivity.

Preparation of antiserums: Rabbit antiserums were prepared against 2 equine viral isolates and used in a cross serum neutralization test between both isolates. Rabbits were given 2 intravenous injections at a week's interval. They were bled 3 weeks after the last injection. Guinea pig antiserums were prepared against the equine isolate, normal EFD cell culture fluid and a canine adenovirus (ICH virus D43 strain). Guinea pigs were given 4 intraperitoneal injections of each cell culture virus and normal cell culture fluid. They were bled 7 days after the final injection.

Serum neutralization (SN) test: The SN tests were carried out in EFD cell monolayers in tubes. In the cross SN test, equal volumes of serum diluted in twofold steps and virus suspension containing approximately 500 $TCID_{50}$ /0.5 ml of infective virus were mixed and incubated at 37°C for 1 hour. While for an antibody survey, serum diluted in tenfold steps and viral suspension of equine isolate diluted to contain 100 $TCID_{50}$ /0.5 ml of infective virus were employed. Each mixture (0.2 ml) was inoculated into cell culture tubes. The antibody titer was expressed as the 50% end point calculated by the method of Reed and Muench [16].

Complement fixation (CF) test: A cross CF test was performed for group-specific antigenicity between a canine adenovirus and the equine viral isolate. Infected cell culture fluid was centrifuged at 3,000 rpm for 10 minutes, and used as antigen. Control antigen was prepared from uninoculated cell culture in a similar manner. The CF antibody titer was expressed as the reciprocal of the highest dilution of serum that reduced hemolysis to 25% or less.

Serologic survey: Eighteen horse serum samples were collected from a stud, from which the colt was derived, in Ibaraki Prefecture in 1976. They were inactivated at 56°C for 30 minutes for the SN test, and at 60°C for 20 minutes for the CF test. An antibody assay of each serum sample was carried out by the SN and CF tests described above.

Results

Virus isolation: Isolation was made in confluent primary horse kidney cell cul-

tures incubated at 37°C. Specimens from the bronchus and pneumonic lesion produced an adenovirus-like cytopathogenic effect (CPE) characterized by foci of refractile, round cells after 6 days. Cover-slip preparations of horse kidney cells stained with May-Grünwald and Giemsa stain were found to contain intranuclear inclusions. Each equine isolate reacted reciprocally in the cross SN test using rabbit antisera raised against each isolate. Thus the isolate from the pneumonic lesion was employed as a representative for further characterization.

Host cell range: As shown in Table 1, the isolate induced CPE in primary kidney cell cultures of horse, pig, fetal pig, dog and rat, and cell lines of PS and EFD. The isolate grew and produced a similar CPE and intranuclear inclusions in EFD cells (Fig. 3), as well as in horse kidney cell culture. No viral replication, however, occurred in cat kidney, Vero, JINET, BHK-21 or HmLu cells.

Nucleic acid type: Treatment of the equine isolate with IUDR resulted in a 3 log unit decrease in the titer, but did not affect the replication of EAV (Table 2).

Sensitivity to chloroform and ether: The infectivity of equine isolate was not affected by the chloroform or ether treatment (Table 3).

pH sensitivity: The isolate remained stable within a range from pH 4.0 to pH 7.0. There was a slight decrease in viral titer after exposure to pH 3.0 for 3 hours (Table 4).

Hemagglutination: The equine isolate, at a titer of $10^{4.0}$ TCID₅₀/0.2 ml, agglutinated human (type O), horse, guinea pig, rat and sheep erythrocytes, but did not pig, dog, cat, hamster, mouse and fowl erythrocytes (Table 5).

Electron microscopy: In thin-sectioned

Table 1. Susceptibility of cell cultures to the equine viral isolate

Cell designation	CPE	Virus titer*
Primary cell cultures		
Horse kidney	+	4.5
Pig kidney	+	NT**
Fetal pig kidney	+	4.5
Dog kidney	+	3.5
Rat kidney	+	3.5
Cat kidney	—	NT
Cell lines		
EFD (Equine fetal dermis)	+	4.5
PS (Pig kidney)	+	NT
Vero (Monkey kidney)	—	NT
JINET (Monkey kidney)	—	NT
BHK-21 (Baby hamster kidney)	—	NT
HmLu (Hamster lung)	—	NT

Remarks.

*: Expressed as negative log₁₀ TCID₅₀/0.2 ml.

**: Not tested.

Table 2. Effect of 5-iodo-2'-deoxyuridine (IUDR) on viral replication

Treatment	Viral titer*	
	Equine viral isolate	EAV**
IUDR	1.5	5.0
Control	4.5	5.5

Remarks.

*: Expressed as negative log₁₀ TCID₅₀/0.2 ml.

**: Equine arteritis virus serving as a control.

Table 3. Effect of chloroform and ether on viral infectivity

Treatment	Viral titer*	
	Equine viral isolate	EAV**
Chloroform	3.74	<1
Control	3.83	4.50
Ether	3.50	<1
Control	3.50	4.83

Remarks.

*, **: See the footnote of Table 2.

preparations, most viral particles were observed with crystalline arrays in the nucleus of the infected EFD cell. Viral particles exhibited a hexagonal configuration and were 64 nm in diameter (Fig. 4). In nega-

Table 4. Effect of pH on infectivity of the equine viral isolate

Medium	pH	Viral titer*			
		0.5 hour		3 hours	
		Equine viral isolate	EAV**	Equine viral isolate	EAV
McIlvaine buffer	2.0	2.74	2.00	1.50	< 1
	4.0	3.24	3.24	3.24	2.50
	6.0	3.24	3.24	3.24	3.24
	7.0	3.24	3.24	3.24	3.24
MEM control	7.3	3.24	3.24	3.24	3.24

Remarks.

*, **: See the footnote of Table 2.

Table 5. Hemagglutinating activity of the equine viral isolate

Species	Temperature	
	4°C	37°C
Human (type 0)	+ (128) *	+ (128)
Horse	+ (16)	—
Guinea pig	+ (16)	—
Rat	—	+ (32)
Sheep	+ (8)	—
Pig	—	—
Dog	—	—
Cat	—	—
Hamster	—	—
Mouse	—	—
Fowl	—	—

Remarks.

*: HA titer is shown in parentheses.

Fig. 1. Distribution of hemagglutinating activity and infectivity of the equine viral isolate in a CsCl gradient

Table 6. Cross complement fixation test between a canine adenovirus (ICH virus D43) and the equine viral isolate

Antiserum against	Antigen			
	Equine viral isolate	ICH virus	EFD control	DK** control
Equine viral isolate	64*	32	< 4	< 4
ICH virus	8	256	< 4	4
EFD cells	< 4	4	< 4	< 4

Remarks.

*: Titer expressed as the reciprocal of the highest dilution of serum that reduced hemolysis to 25% or less.

**: Dog kidney cell culture.

Table 8. Serologic survey on the stud from which the foal was derived

No.	Horse			SN antibody titer**	CF antibody titer***
	Breed	Age	Sex		
1	Thoroughbred	11 years	stallion	3,162	<4
2	Thoroughbred	16 years	mare	3,162	8
3	Thoroughbred	15 years	mare	3,162	8
4	Thoroughbred	15 years	mare	3,162	8
5*	Thoroughbred	12 years	mare	3,162	8
6	Thoroughbred	10 years	mare	3,162	8
7	Thoroughbred	7 years	mare	316	4
8	Thoroughbred	5 years	mare	316	<4
9	Arab	16 years	gelding	3,162	8
10	Breton	4 years	gelding	316	4
11	Halfbred	4 years	gelding	316	4
12	Arab	3 years	filly	316	4
13	Thoroughbred	2 years	filly	1,778	4
14	Thoroughbred	1 year	colt	17	<4
15	Thoroughbred	1 year	filly	3,162	4
16	Thoroughbred	5 months	filly	32	<4
17	Thoroughbred	5 months	colt	32	<4
18	Thoroughbred	3 months	filly	32	<4

Remarks.

*: The dam of the foal investigated.

**: Expressed as the reciprocal of dilution at 50% end point.

***: Expressed as the reciprocal of the highest dilution of serum that reduced hemolysis to 25% or less.

Table 7. Antigenic relationship of the equine viral isolate and a canine adenovirus (ICH virus D43) as determined by the serum neutralization (SN) test

Antiserum against	SN antibody titer*	
	Equine viral isolate	ICH virus D43
Equine viral isolate	2,951	<20
ICH virus D43	<20	1,499

Remarks.

*: Expressed as the reciprocal of dilution at 50% end point.

tive staining, they had a cubic symmetry without envelope. The equine isolates were 74 nm in average diameter (Fig. 6).

Buoyant density: A sharp band was formed at a density of 1.29 g/cm³. It was substantiated by infectivity and hemagglutinating activity (Fig. 1).

Serologic properties of the equine isolate: The cross CF test indicated that a common CF antigen was shared between a canine adenovirus and the equine viral isolate (Table 6). In the cross SN test, however, the equine isolate was antigenically distinct from a canine adenovirus (Table 7).

Serologic survey on the stud: Of the 18 horse serum samples, 14 had a SN antibody titer of 1:100 or higher when tested against the equine isolate. As a rule, frequency of antibody was lower in foals from 3 months to 1 year of age than in horses over 1 year of age. The actual SN and CF antibody titers of horses in the stud are indicated in Table 8.

Discussion

Two cytopathogenic agents were isolated in horse kidney cell cultures from the bron-

chus and pneumonic lung tissue of a three-month-old Thoroughbred colt. They produced an adenovirus-like CPE accompanied with the formation of intranuclear inclusions in the EFD cell line, as well as in the primary horse kidney cell culture. The isolate from the pneumonic lesion could be used as a representative for characterization, since both isolates reciprocally reacted in the cross SN test.

The biological and physicochemical properties of the equine isolate described in the present paper show that the isolate is an adenovirus. Viral particles in negative staining had a cubic symmetry without envelope. They were 74 nm in average diameter. Treatment with IUDR inhibited their replication, suggesting that the virion might contain DNA. The isolate was not susceptible to chloroform or ether, indicating that the virus lacked essential lipids. It was stable within a range from pH 4.0 to pH 7.0, but was slightly inactivated when held at pH 3.0 for 3 hours. The equine adenoviruses reported by Ardans et al. [2] and Dutta [5] were stable at pH 3.0, while that reported by Harden [8] was inactivated at pH 3.0.

The isolate was propagated in not only horse kidney cells but also pig kidney, fetal pig kidney, dog kidney, rat kidney and PS cells. It did not grow, however, in cat kidney, Vero or BHK-21 cells. The strain isolated by Harden et al. [9] was reported to be propagated in cat kidney, Vero and BHK-21 cells. England et al. [6] demonstrated that their isolate grew neither in dog kidney cells nor in BHK-21 cells. In this respect the host cell range of equine adenovirus seems to be unsettled.

The isolate agglutinated human (type O), horse, guinea pig, rat and sheep erythrocytes, but did not pig, cat, dog, hamster, mouse or fowl erythrocytes. Horse erythro-

cytes were agglutinated by the adenovirus isolated by McChesney et al. [12] and Wilks and Studdert [21], but not by those isolated by Petzoldt and Schmidt [15] and Dutta [5]. The isolate reported by England et al. [6] caused hemagglutination of mouse erythrocytes, but the isolate by Dutta [5] or by the present authors did not. The buoyant density of the equine isolate was 1.29 g/cm³ in CsCl. The buoyant density of equine adenovirus was reported to be 1.27, 1.31 and 1.33 g/cm³ by England et al. [6], Ardans et al. [2] and Harden [8], respectively. The lower buoyant density of equine adenovirus may be attributed to the low ratio of nucleic acid to protein in the virion.

Common group-specific antigen was detected by the cross CF test between the isolate and a canine adenovirus. Anti-ICH virus guinea pig serum reacted weakly with equine viral antigen. This serologic relationship is similar to that between ICH virus and equine adenovirus described by Wilks and Studdert [21], and that between ICH virus and human adenovirus described by Carmichael and Barnes [3].

In a serologic survey high levels of SN antibody against the isolate were detected in aged horses. They were interpreted as evidence of long-standing enzootics in the stud. As a matter of fact, respiratory disease has often been observed among the horse of the stud. It is not clear whether it is adenoviral infection or not. In the present case, it is considered that adenovirus may have acted as trigger for fatal bacterial infection.

Pathogenicity of adenovirus for horses has not been known as yet, but it was recognized that an Arabian foal with immunodeficiency died of adenovirus infection [4, 13]. The present paper may be the first to report the occurrence of fatal adeno-

viral infection in non-Arabian foals.

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Addendum: After submitting the manuscript, the "Australia '75" strain of equine adenovirus isolated by Dr. Timoney was kindly supplied by the Equine Health Laboratory, Japan Racing Association, which was found to be identical to authors' isolate in the reciprocal serum neutralization test.

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Explanation of Figures

Fig. 2. Normal EFD cells 4 days after seeding. May-Grünwald and Giemsa (MGG) staining, $\times 300$.

Fig. 3. Intranuclear inclusions produced in EFD cells by the equine isolate 5 days after inoculation. MGG staining, $\times 300$.

Fig. 4. Crystalline lattice arrangement of viral particles in the nucleus of EFD cells. Uranyl

acetate-lead nitrate staining, $\times 18,000$.

Fig. 5. High-power magnification of the crystalline array of viral particles in Fig. 4. Uranyl acetate-lead nitrate staining, $\times 100,000$.

Fig. 6. Morphology of viron of the equine viral isolate. Phosphotungstic acid staining, $\times 160,000$.