Genetic Characterization of Fowl Adenoviruses Isolated from Chickens with Hydropericardium Syndrome in Japan

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ABSTRACT. We genetically characterized fowl adenoviruses (serotype 4 FAdV, FAdV-4) isolated from chickens with hydropericardium syndrome (HPS) in Japan by the polymerase chain reaction (PCR) method coupled with direct sequencing. Phylogenetic analysis based on the part of the hexon gene that included the L1 region revealed that all FAdV-4 isolates from chickens with HPS in Japan were identical and were distinguished completely from the cluster including FAdV strains from chickens with HPS in India and Pakistan. This suggested that FAdV-4 from the HPS chickens in India and Pakistan was derived from a common ancestor, but the origin of the FAdV-4 from the HPS chickens in Japan was completely different.

KEY WORDS: fowl adenovirus, hydropericardium syndrome, phylogenetic analysis.

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Hydropericardium syndrome (HPS) is a comparatively newly emerging disease of chickens that was initially reported in Pakistan in 1987 [6]. It is also called “Angara disease”, since it first occurred in Angara Goth, Karachi, Pakistan in August 1987. HPS was later observed in Japanese quail (*Coturnix coturnix japonica*) [16]. HPS is seen primarily in broilers at age 3 to 5 weeks, although outbreaks have been reported in layers and breeders [4]. The disease is characterized by an accumulation of near-amber fluid in the pericardial sac and an enlarged discolored liver with foci of hemorrhage and/or necrosis. Histologically, intranuclear, basophilic inclusion bodies are visible in the hepatocytes. The syndrome has been reproduced in broilers by the inoculation of a liver homogenate from affected birds. An adenovirus that was either isolated from or visualized by electron microscopy in the livers of affected chickens has been implicated in the syndrome, and the syndrome has been reproduced by inoculation of the isolated virus [8, 15]. Therefore, the syndrome is also called infectious hydropericardium.

So far, HPS has been reported in Pakistan, Japan, Iraq, Kuwait, India, the former U.S.S.R. Chile, Mexico and Korea [1, 2, 7, 9, 11, 13, 19]. In Japan, the first outbreak of HPS was confirmed in Tokushima prefecture, in the western part of Japan, in 1996 [2]. Thereafter, sporadic outbreaks were observed in Tokushima prefecture and neighboring regions. Fowl adenoviruses (FAdV) were isolated from all of the cases in these outbreaks. Here, we describe the genetic properties of the viruses isolated from these outbreaks to gain an understanding of the epidemiology of this virus.

MATERIALS AND METHODS

Viruses: The case history of the HPS outbreaks in chickens in Japan used in this study is summarized in Table 1. Primary chicken kidney cell cultures (CKC) were routinely used for isolation and propagation of the virus. Livers from chickens naturally affected by HPS between 1996 and 2001 were ground with sterile sand and diluted with maintenance medium to make a 10% (w/v) suspension. The homogenates were then centrifuged at low speed ($3,000 \times g$ for 10 min) for virus isolation. CKC were inoculated with the supernatants, incubated at 37°C for 5 days, and observed daily for cytopathic effects (CPE). The presence of FAdV in CKC was initially determined by immunofluorescence assay of cells using anti-FAdV chicken serum.

The old conventional FAdV-4 strains used in this study, KR-5, 828-3, 1078-5, 1081-2 and 1262-4, were previously isolated by Kawamura *et al.* in Japan and maintained at our institute. Among them, the KR-5 strain is the reference strain of FAdV-4 [10].

The FAdV isolate from a chicken with HPS, H-1 strain, in India was kindly provided by Dr. R. Manickam (Tamil Nadu Veterinary and Animal Sciences University, Madhavaram Milk Colony Campus, Chennai, India). The FAdV isolates from chickens with HPS in Pakistan, PARC-1/96, PARC-1/97, and PARC-1/98 strains were kindly provided by Dr. M. Qasim Khan (Animal Health Institute, National Agricultural Research Centre, Park Road, Islamabad, Pakistan).

Virus neutralization test: Serotyping was performed by micro-neutralization methods [14]. Briefly, the constant virus and varying serum technique was employed in which two hundred 50% tissue culture-infected doses (TCID₅₀) of virus isolate and twofold dilutions of antisera, starting from 1:20, were used. Two wells were used per virus-serum mix-

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Table 1. Summary of HPS cases in Japan used in this study

Prefecture	Year	Type of chicken	No. birds in flock	Age at onset (days)	Mortality (%)	Name of isolated virus
Tokushima	1996	Broiler breeder	5,000	240–260	6.4	JP/LVP-1/96
Tokushima	1996	Commercial broiler	11,220	14–28	20.2	JP/Tokushima/1/HPS/96
Tokushima	1996	Commercial broiler	34,145	14–37	26.1	JP/Tokushima/2/HPS/96
Kagawa	2000	Commercial broiler	4,000	13–17	10	JP/Kagawa/HPS/2000
Ehime	2001	Commercial broiler	17,000	14	27.1	JP/Ehime/HPS/2001

ture. The neutralization titers were read one week after inoculation, and the reciprocal of the highest dilution of the serum showing a 50% endpoint of neutralization was used. Antisera to each of the eight conventional FAdV reference serotype strain prepared in a previous study [10] were used. Convalescent serum to the JP/LVP-1/96 strain obtained from a previous study [15] was used as an antiserum to the FAdV isolated from chickens with HPS.

Polymerase chain reaction (PCR): To determine the genetic relationship between the study HPS-FAdV-4 strains and other viruses, we determined partial nucleotide sequences of the hexon gene of the isolates. Viral DNAs from infected culture fluids or tissue homogenates were extracted using the QIAamp DNA Micro Kit (Qiagen Inc., Valencia, CA, U.S.A.). The primers were designed from conserved reported sequences identical to a region of the hexon protein gene of several FAdVs including the L1 region. The following primer set [12] was used in this study: HexF1: 5'-GAYRGYHGGRTNBTGGAYATGGG-3' (sense) and HeXR1: 5'-TACTTATCNACRGCTGRTTCCA-3' (antisense). PCR was performed using 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. The predicted size of the PCR products was approximately 800 bp.

Nucleotide sequencing and phylogenetic analysis of FAdV-4 strains: To examine the relationship among FAdV-4 strains, we directly determined the nucleotide sequences of the generated PCR products. The obtained PCR products of FAdV-4 were purified with Montage (Millipore, Biller-

ica, MA, U.S.A.), according to the manufacturer's instructions. The purified PCR products were used as a template for sequencing on an Applied Biosystems 3100 automated DNA sequencer using dye terminator cycle sequencing chemistry (Applied Biosystems, Foster City, CA, U.S.A.). The purified PCR products were sequenced from both directions.

Sequences of the FAdV-4 strains were aligned with the ClustalX program [18], and phylogenetic trees were constructed by the neighbor-joining (NJ) method [17].

PCR-restriction fragment length polymorphism (PCR-RFLP) analysis: The generated PCR products were employed in restriction endonuclease analysis for the development of a simple and rapid classification of the FAdV-4 strains. After comparing all the sequences obtained in this study (GENETYX-Mac ver. 13 Software Development Corp., Tokyo, Japan), we found that *Mbo* II (TaKaRa, Tokyo, Japan) worked best for classification among FAdV-4 strains.

RESULTS

Serotype of FAdV isolates from affected chickens with HPS (HPS-FAdV-4): All isolates from the five cases of HPS were neutralized by FAdV-4 (KR-5) antiserum (Table 2). No major antigenic differences were observed in the results of the cross-neutralization test.

Nucleotide sequencing and phylogenetic analysis of FAdV-4 strains: The primer pair for the PCR (HexF1 and

Table 2. Results of the cross-neutralization test among HPS-FAdV strains

Viruses	Antiserum								
	1(OTE)	2(SR-48)	3(SR-49)	4(KR-5)	5(TR-22)	6(CR-119)	7(YR-36)	8a(TR-59)	JP/LVP-1/96
KR-5 (type 4 reference)	–	–	–	2560	–	–	–	–	5120
JP/LVP-1/96	–	–	–	5120	–	–	–	–	10240
JP/Tokushima/1/HPS/96	–	–	–	2560	–	–	–	–	2560
JP/Tokushima/2/HPS/96	–	–	–	2560	–	–	–	–	10240
JP/Kagawa/HPS/2000	–	–	–	2560	–	–	–	–	2560
JP/Ehime/HPS/2001	–	–	–	2560	–	–	–	–	5120
H-1(India)	–	–	–	2560	–	–	–	–	1280
PARC-1/96(Pakistan)	–	–	–	2560	–	–	–	–	1280
PARC-1/97(Pakistan)	–	–	–	2560	–	–	–	–	5120
PARC-1/98(Pakistan)	–	–	–	2560	–	–	–	–	2560

–: <20.

Fig. 1. Phylogenetic tree based on the hexon gene of FAdV4. Nucleotides 381–1102 (722 bases) of the hexon gene of FAdV4 (GenBank Accession No. EU177544) were subjected to phylogenetic analysis. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. The viruses employed in this study are italicized and underlined.

HexR1) successfully amplified products of about 800 bp from the DNA samples of all FAdV strains employed in this study. The nucleotide sequences of all HPS-FAdV-4 strains in Japan from 1996 to 2001 were completely identical. According to phylogenetic analysis based on the hexon gene with available sequences from GenBank, the HPS-FAdV-4 strains in Japan belonged to the same cluster, and were completely different from the FAdV-4 isolates from the HPS-FAdV-4 strains in India/Pakistan (Fig. 1). The old conventional FAdV-4 strains also belonged to a different cluster. Interestingly, many old conventional FAdV-4 strains were belonged to a different cluster than KR-5, which was the reference strain of serotype 4 of FAdV.

PCR-RFLP analysis: The FAdV-4 strains were distinguished by the *Mbo* II restriction profiles (Fig. 2, Table 3). The Japanese HPS-FAdV-4 strains showed a different profile than HPS-FAdV-4 in the India/Pakistan strains derived from HPS and than the old conventional FAdV-4 strains.

DISCUSSION

HPS was initially reported in Pakistan in 1987 [6], and thereafter outbreaks of this syndrome were reported in other Middle Eastern countries, as well as in Latin America and Japan. However, the epidemiological relationship of FAdV isolated from poultry with HPS in these countries is not fully understood.

In this study, we genetically analyzed FAdV isolated from chickens with HPS. Phylogenetic analysis of the hexon gene revealed that the HPS-FAdV-4 strains in Japan belonged to different clusters than those from India and Pakistan. HPS-FAdV-4 in India and Pakistan seemed to be derived from a common ancestor. Among the old conventional FAdV-4 strains collected by Kawamura *et al.* in Japan, interestingly, the reference FAdV-4 strain, KR-5, was located in a different cluster than the other strains. Con-

Fig. 2. Restriction endonuclease analysis of the PCR-amplified hexon genes from the four representative genetic groups of FAdV-4 digested with *Mbo* II. Lane M: molecular weight marker Φ X174-*Hae* III digest, Lane 1: KR-5, Lane 2: 1078-5 (Old conventional serotype 4), Lane 3: JP/LVP-1/96, Lane 4: PARC-1/96.

sidering that the HPS-FAdV-4 strains were classified into five types by restriction endonuclease analysis of infected cellular DNA in a previous report [9], the FAdV-4 related to HPS seemed to originate independently from multiple viruses. Further genetic analysis, particularly determination of the nucleotide sequences, of FAdV-4 from chickens with HPS isolated in other regions is required to gain a better understanding of the epidemiology of FAdV related to HPS.

The PCR-RFLP analysis using *Mbo* II would be useful for characterizing the FAdV isolated from chickens with HPS for the classification of FAdV-4 strains. Although sequencing is recommended to obtain precise genetic information, PCR-RFLP analysis is simple, easy and convenient for primary characterization in routine diagnosis.

Although a commercial vaccine against HPS-FAdV-4

Table 3. Predicted size of PCR-RFLP analysis by *Mbo* II

Type	Viruses	Predicted fragment size (bp)
Reference strain of type 4	KR-5	530/249
Old conventional FAdV4 strains in Japan	828–3,1081–2,1262–4	295/243/235/6
Old conventional FAdV4 strains in Japan	1078–5	295/249/235
HPS-FAdV4 strains in Japan	JP/LVP-1/96 JP/Tokushima/1/HPS/96 JP/Tokushima/2/HPS/96 JP/Kagawa/HPS/2000 JP/Ehime/HPS/2001	489/249/41
HPS-FAdV4 strains in India and Pakistan	H-1 PARC-1/96 PARC-1/97 PARC-1/98	489/243/41/6

has not been developed, studies have demonstrated that the inoculation of inactivated liver was efficient to protect against this HPS [3, 5]. The result of the cross-neutralization test in this study revealed no major antigenic diversity among FAdV-4 strains. Therefore, if HPS becomes epidemic, application of inactivated vaccine would be the primary means of controlling of the disease. However, molecular analysis of HPS-FAdV-4 would provide useful information about the development of an efficient vaccine against HPS and the mechanism of emergence of highly pathogenic FAdV strains.

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