Epidemiological studies on fowl adenoviruses isolated from cases of infectious hydropericardium

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Epidemiological studies on fowl adenoviruses isolated from cases of infectious hydropericardium

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Serology, restriction enzyme analysis and polymerase chain reactions were used to classify a total of 12 fowl adenoviruses (FAV) isolated from clinical cases of infectious hydropericardium from field outbreaks in seven countries in Asia and America. All isolates belonged to FAV serotype 4. Two isolates were contaminated with avian adeno-associated virus and one of them also contained FAV1. Minor differences were observed in the *Bam*HI restriction profiles. More variability was seen with *Sma*I, *Bgl*II and *Pst*I restriction profiles. However, more than 80% of the fragments were identical in size in the five different *Pst*I profiles, indicating the close genomic relationship between the isolates. Polymerase chain reaction assays supported the classification of the isolates as FAV4 strains. All isolates could be detected using H1/H2 or H3/H4 primer pairs. Restriction enzyme analysis of the H1/H2 polymerase chain reaction (PCR) products allowed no differentiation between the isolates, whereas the three isolates from India and Pakistan could be separated from all others after *Hpa*II digestion of the H3/H4 PCR products. Although strain variation was demonstrated, it could be shown that all adenoviruses isolated from various field cases of infectious hydropericardium (Angara Disease) in several countries belong to fowl adenovirus serotype 4.

Introduction

The first occurrence of infectious hydropericardium (IHP) was reported in Angara Goth in Pakistan in late 1987 (Anjum *et al.*, 1989). The disease is also called Angara Disease or Hepatitis/Hydropericardium Syndrome. Spread of the disease was reported in India, Iraq and Kuwait. On the American continents, the disease was reported in Mexico, Peru, Ecuador and Chile (Shane & Jaffery, 1997). Based on electron microscopic data, it was concluded that IHP is caused by an adenovirus infection (Cheema *et al.*, 1989; Chandra *et al.*, 1997). The first isolation of an adenovirus from field cases was reported by Voß (1989). Recently, we described the successful reproduction of the disease in different aged specific pathogen free (SPF) chicks using fowl adenovirus (FAV) serotype 4 strains isolated from Pakistan and Ecuador (Mazaheri *et al.*, 1998). In these investigations, genomic differences were found between the isolates from Pakistan and Ecuador. The isolation and characterization of FAV4 isolates from field outbreaks in Chile was described by Cowen *et al.* (1996) and Toro *et al.* (1999). These results indicated that some FAV4 strains play a major role in the aetiology of IHP.

The aim of this study was to type and classify adenoviruses isolated from field cases of IHP in different countries using serotyping, restriction enzyme analysis (REA) of the whole genome and polymerase chain reaction assays combined with REA of hexon gene fragments.

Material and Methods

Virus isolation

All 12 isolates were grown in chicken embryo liver cells from 11-day-old SPF embryos (Valo; Lohmann Tierzucht GmbH, Cuxhaven, Germany). For cell propagation, Medium 199 containing Earles salts and 10% fetal calf serum was used. Table 1 summarizes the field isolates included in this study.

Serotyping

Serotyping was done by serum neutralization test in microtiter plate cultures of chicken embryo liver cells as described by Monreal *et al.* (1980). Reference sera against FAV1 to FAV12 containing 20 U antibodies together with 100 tissue culture infectious doses, median (TCID50) of each virus were used for typing the isolates (McFerran, 1989).

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Table 1. *Description of the IHP isolates included in this study and grouping of the isolates into five genome types together with the FAV4 reference strain KR5 after restriction enzyme analysis*

Lane on agarose gels	Virus	Country[a]	Chickens	Year of isolation	Genome type[b]
1	KR5 = FAV4 reference strain				I
2	IV4	Mexico	Broiler	1995	II
3	K388	Mexico	Broiler	1995	II
4	K1013–1	Ecuador	Broiler	1995	III
5	K1013–3	Ecuador	Broiler	1995	III
6	P53	Peru	Broiler	1997	III
7	P54	Peru	Broiler	1997	III
8	C344	Chile	Broilerbreeder	1996	III
9	K99–5	Kuwait	Broiler	1997	IV
10	K99–7	Kuwait	Broiler	1997	IV
11	K31	Pakistan	Broiler	1989	V
12	K88	Pakistan	Broiler	1995	V
13	IV37	India	Broiler	1996	V

[a] Isolates from the same country came from different farms.
[b] Genome type according to *Pst*I restriction profiles (Figure 2).

DNA extraction and restriction enzyme analysis

Isolates were grown in embryo liver cells for 3 to 4 days until greater than 80% cytopathic effect could be seen. After three freeze–thaw cycles, 100 ml infected cells were mixed with 50ml chloroform and centrifuged for 20 min at 4000x*g* to remove cellular debris. The supernatant was centrifuged at 100 000x*g* for 90 min and the pellet was resuspended in Tris-ethylenediaminetetraacetic acid (EDTA) buffer containing 1 mg proteinase K/ml. DNA was purified by phenol/chloroform extraction and precipated with ethanol. DNA content was measured fluorometrically with the VersaFluor™ Fluorometer System (Bio-Rad Laboratories). Briefly, lambda DNA was used for calibration together with bisbenzimide. Measurement was performed with the filter set EX 360/40 and EM 460/10 as described in the manual. Purified FAV DNAs were digested with the restriction endonucleases *Hin*dIII, *Bam*HI, *Bgl*II, *Pst*I and *Sma*I, as recommended by the manufacturer (Boehringer Mannheim, Mannheim, Germany). Cleavage patterns for *Pst*I were classified as patterns I to V. Graphic alignments of the *Pst*I profiles were done with the BIO-Profil 1D V97 software package using the Dice coefficient of similarity as follows: two times the number of matching bands/total number of bands in both strains (Dice, 1945). The confidence value was set at 4%.

Polymerase chain reactions

Polymerase chain reactions (PCR) were conducted using the primer pairs H1/H2 and H3/H4 (Raue & Hess, 1998). After DNA amplification, PCR products were separated by electrophoresis in 0.5% agarose gel containing 0.5 μg ethidium bromide/ml. FAV4 reference strain KR5 was included as a control in the PCR and subsequent restriction enzyme digestions.

The PCR fragments produced after amplification by the H1/H2 primer set were cleaved with the restriction enzyme *Hae*II and those produced by primer set H3/H4 were cleaved with *Hpa*II according to the manufacturer's recommendations (Boehringer Mannheim). The restriction fragments were separated by agarose gel electrophoresis (3% NuSieve-GTG agarose containing 1% SeaKem-GTG agarose; FMC, Germany) at 19 V for 18 h. Fragment sizes were compared with commercially available standard molecular weight markers (Life Technologies).

Results

Serum neutralization test

All isolates were neutralized by FAV4 (KR5) and FAV4 (K31) antisera. Some isolates exhibited some cross-reactivity with FAV11 (C2B) antisera. None of the other FAV antisera reacted with any of the isolates.

Restriction enzyme analysis

In order to classify the isolates, they were digested with *Hind*III (data not shown) and *Bam*HI, and compared with published results for FAV4. With these enzymes, all isolates and the FAV4 reference strain KR5 had similar restriction endonuclease digest profiles (Figure 1). Only the two Mexican isolates (lanes 2 and 3) showed some variations in the *Bam*HI restriction profile. The *Sma*I restriction profiles of the American isolates were identical, whereas the isolates from Asia produced different restriction profiles. Among the Asian isolates, those from India and Pakistan were identical but they differed from the two isolates from Kuwait. After digestion of the genomes with *Bgl*II, all isolates from Asia were identical. The two isolates from Mexico could be differentiated from all Asian and the other American isolates. Differences between the Mexican isolates and all others were seen with all restriction enzymes except *Sma*I. Most variation was seen in the *Pst*I restriction profiles, which were labelled I to V (Figure 2). The reference strain KR5 was the sole member of genome type I. Type II contained the isolates from Mexico, type III the isolates from Ecuador, Peru and Chile, type IV the isolates from Kuwait, and type V the isolates from Pakistan and India. However, for each enzyme including *Pst*I, the differences between the genome types were limited to one or two cleavage sites. The identity between the isolates was investigated by determining the number of pairwise comigrating restriction fragments

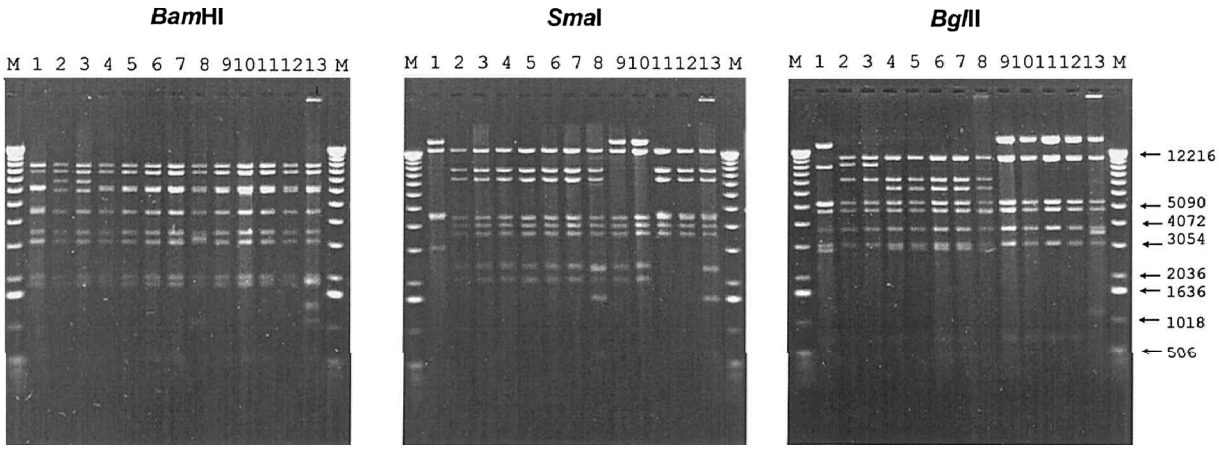

Figure 1. *Cleavage patterns of genomic DNA of KR5 (lane 1) and IHP field isolates (lanes 2 to 13) digested with BamHI, SmaI and BglII. M, 1 kb DNA-marker (Life Techonologies).*

of the *Pst*I digests. The dendrogram in Figure 2(b) shows that more than 80% of the fragments were shared between the isolates, although *Pst*I was the enzyme with the greatest variability (Figure 2(b)). In addition, the dendrogram shows the clustering of the reference strain and field isolates into five different genome types (I to V).

Two of the isolates (C344, lane 8; and IV37, lane 13) had additional bands in the restriction profiles which should be interpreted with some caution. These bands were identified as contamination of the respective strains with avian adeno-associated virus (AAAV) by Southern hybridization (data not shown) and the bands correspond to different forms of AAAV DNA (Hess *et al.*, 1995).

Polymerase chain reactions

A single 1219 bp fragment from the hexon gene was amplified from all isolates and the reference strain KR5 using the H1/H2 primer pair (Figure 3(a)). The *Hae*II digest of the PCR products generated two fragments of approximately 0.2 and 1 kb for all samples (Figure 3(b)). Two additional bands were identified from sample C344. This sample was shown to be contaminated with FAV1 (data not shown) and these extra bands were consistent with this finding. The H3/H4 primer pair amplified a fragment of 1319 bp from all isolates (Figure 4(a)). After *Hpa*II digestion of the PCR products, fragments were produced which ranged from 0.1 to 0.5 kb (Figure 4(b)). The three isolates from India and Pakistan (lanes 12 to 14) showed some variation. The PCR fragments of these isolates lacked one *Hpa*II cleavage site, which resulted in a double band located at 0.5 kb. Contamination with FAV1 was visible in the sample C344 (lane 9).

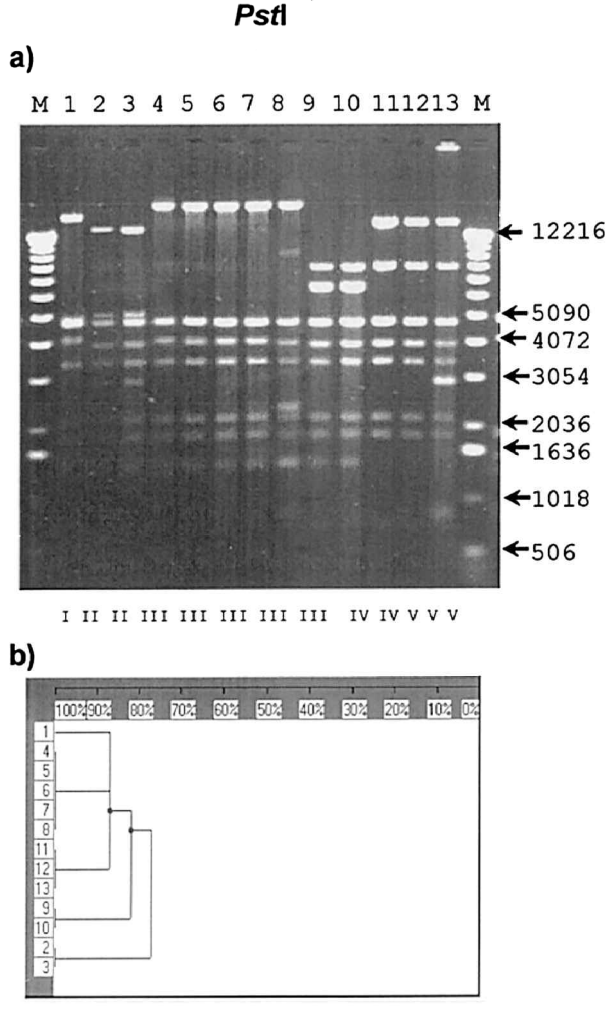

Figure 2. *(a) Cleavage patterns of genomic DNA of KR5 (lane 1) and IHP field isolates (lanes 2 to 13) digested with PstI. Identified genome types (I to V) for each digestion are indicated below agarose gels. (b) Dendrogram showing the relationship between the PstI restriction patterns using the BIO-Profil 1D V97 software package. Whereas the identity values between the restriction profiles is given on the* x *axis, the* y *axis shows the lane number of the corresponding agarose gel.*

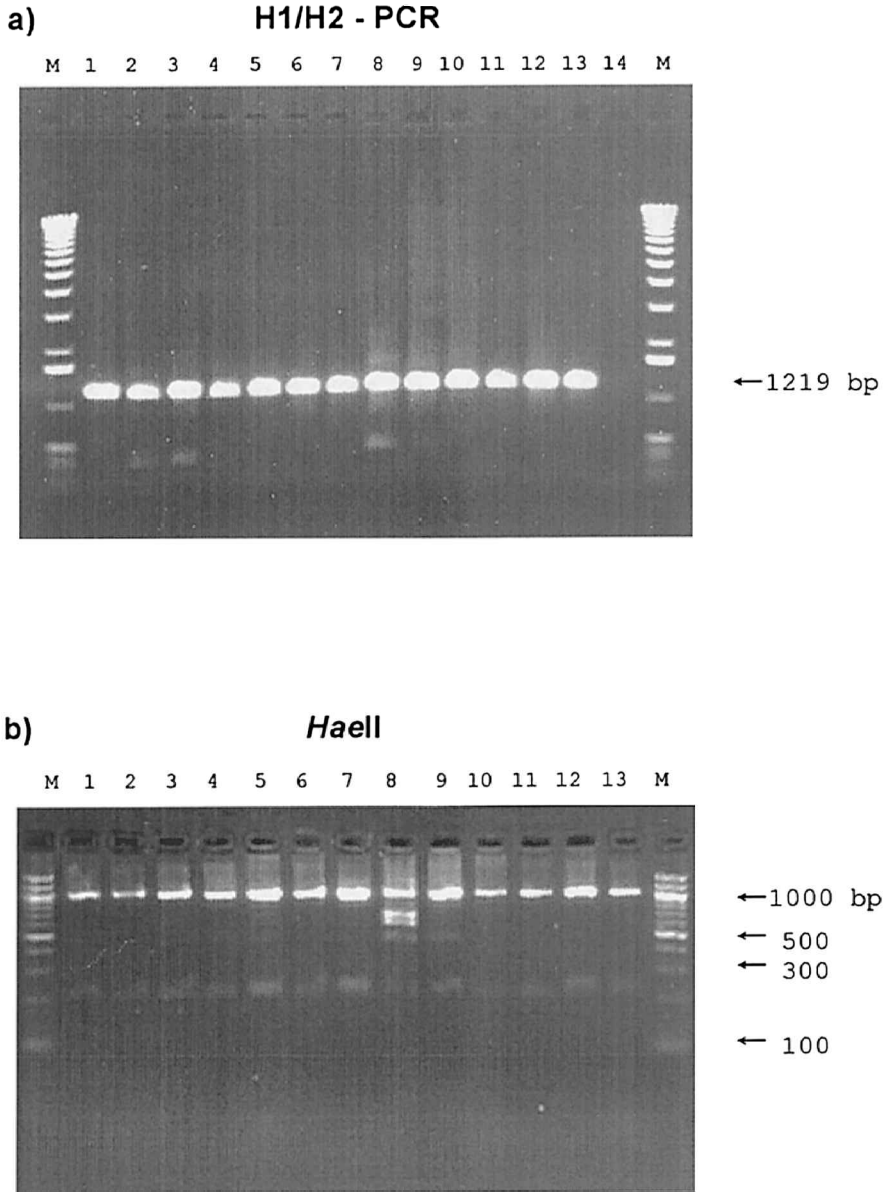

Figure 3. *(a) Electrophoresis of the H1/H2 PCR products from KR5 (lane 1) and field isolates (lanes 2 to 13); lane 14, negative control; M, 1 kb DNA-marker. (b) Separation after HaeII digestion. M, 100 bp DNA-marker (Life Technologies).*

Discussion

IHP is a new disease affecting mainly broiler chickens and has great economic impact in different regions of the world. Recently, we reproduced the disease with purified FAV4 in SPF chicks (Mazaheri *et al.*, 1998). In the present study, we investigated isolates from different countries to gain more information about the epidemiology of the disease. Based on neutralization tests, all isolates belonged to FAV4. Further investigations of the field isolates allowed classification of the isolates into one of the five established FAV DNA groups (A to E) after *Hind*III and *Bam*HI digestion of the genome (Zsak & Kisary, 1984). All isolates were identified as members of DNA group C. The close relationship between the isolates and the FAV4 reference strain KR5 supported their serological classification as FAV4 strains.

The genome size of all isolates was around 44 kb, which is in the range of the completely sequenced FAV1 genome (Chiocca *et al.*, 1996). The restriction enzyme cleavage patterns of the FAV4 reference strain KR5 were almost identical to those published for the CFA15 strain of FAV4 (Erny *et al.*, 1995). To further estimate the relationship between the isolates, *Sma*I, *Bgl*II and *Pst*I restriction digestions were performed, and for *Pst*I, the ratio of identical/total bands was determined. The results of this study indicate that highly pathogenic FAV4 strains belong to the same cluster, although variations in genome types exist. It should be noticed that the isolates from Ecuador, Peru and Chile were identical, showing the same restriction profile for each enzyme. Similarly, the three isolates from Pakistan and India were identified. The two isolates from Mexico were also

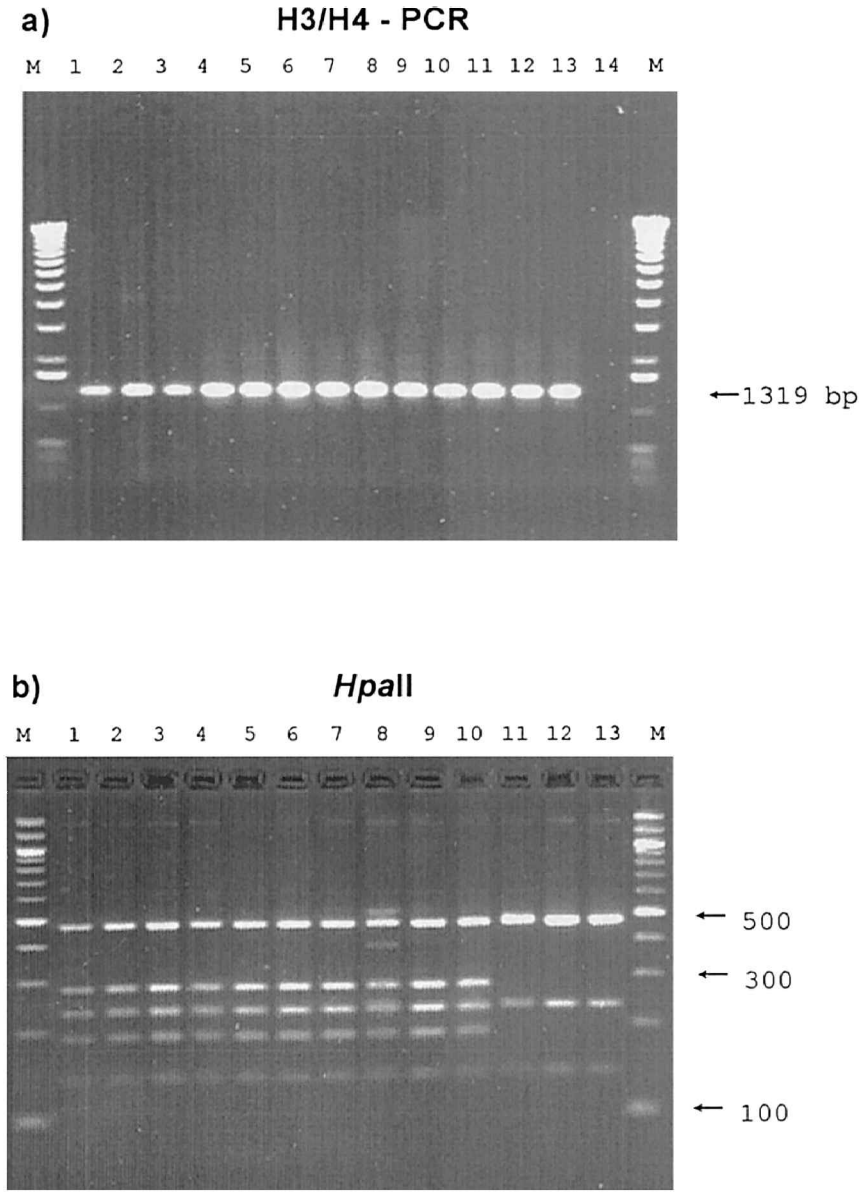

Figure 4. *(a) Electrophoresis of the H3/H4 PCR products from KR5 (lane 1) and field isolates (lanes 2 to 13); lane 14, negative control; M, 1 kb DNA-marker. (b) Separation after HpaII digestion. M, 100 bp DNA-marker.*

identical but different to all others. This observation was also found for the two isolates from Kuwait.

The graphic alignments of this comparison showed that the isolates and the FAV4 reference strain shared more than 80% of their restriction fragments. An 80% value for pairwise comigrating restriction fragments (PCRF) was taken as borderline to divide 40 human adenovirus serotype 7 strains representing 15 genotypes into three different clusters (Li & Wadell, 1986). PCRF values of 93 to 99% were found on comparing highly pathogenic FAVs isolated from IBH cases in Australia belonging to serotypes 7 and 8 (Erny *et al.*, 1991). In these investigations, lower PCRF values (54 to 73%) were able to distinguish between virulent and non-virulent IBH isolates.

The presence of the avian adeno-associated virus in isolates of avian adenoviruses is a common problem (El Mishad *et al.*, 1975). Consequently, if additional fragments up to 4.8 kb occur during REA of FAVs, the results should be interpreted carefully and adenovirus DNA must be checked prior to digestion.

With PCR, all isolates were typed as FAV4 strains. In addition, one isolate from Chile (C344) was shown to be contaminated with FAV1. This contamination reflects the field situation with wide distribution of AAAV and the coinfection of chicks with different FAV serotypes (Erny *et al.*, 1995). The *Hpa*II digest of the H3/H4 PCR products allowed some differentiation between the isolates due to the lack of one *Hpa*II cleavage site in the PCR products amplified from the isolates of

India and Pakistan. Although the *Hpa*II restriction profiles varied slightly between the isolates, the results presented in this study demonstrated the utility of recently established PCRs for detection of FAVs. A further advantage of PCR compared with other diagnostic methods should be mentioned. The presence of two different FAVs in one isolate was only detectable by PCR. The propagation and passaging of field isolates in tissue cultures may lead to selective enrichment of a specific strain, which is then diagnosed by current conventional diagnostic methods. In the future, organ material should be used for PCR prior to isolation of the virus to circumvent this problem. The fact that contamination of C344 with AAAV did not influence the PCRs is an additional advantage of this diagnostic method under field conditions.

In general, the epidemiological data support our earlier results that some strains of fowl adenovirus serotype 4 are the etiological agents of IHP (Mazaheri *et al.*, 1998). Infectious hydropericardium is a disease caused by FAVs belonging to serotype 4. The aim of future studies will be to investigate more isolates to establish the close genomic relationship between the highly pathogenic FAV4 strains.

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RÉSUMÉ

Etudes épidémiologiques concernant les adénovirus aviaires isolés à partir des cas d'hydropéricardite infectieuse[rsh]La sérologie, les profils de restriction et la réaction d'amplification en chaîne par polymérase (PCR) ont été utilisés pour classer 12 souches d'adénovirus aviaires (FAV) isolées de cas cliniques d'hydropéricardite infectieuse (IHP) observés dans 7 pays d'Asie et d'Amérique. Tous les isolats appartenaient au FAV sérotype 4. Deux isolats étaient contaminés par un virus aviaire adénovirus-associé et l'un d'entre eux contenait également un FAV1. Des différences mineures ont été observées dans les profils de restriction de *Bam*HI. Davantage de différences ont été observées dans les profils de restriction de *sma*I, *Bgl*II et *Pst*I. Cependant, plus de 80% des fragments présentaient des tailles identiques dans les 5 profils de *Pst*I indiquant des relations génomiques très proches entre les isolats. Les tests PCR ont confirmé qu'il s'agissait bien de souches FAV4. Tous les isolats ont pu être détectés en utilisant les paires d'amorces H1/H2 ou H3/H4. L'analyse des profils de restriction enzymatique des produits amplifiés par H1/H2 n'a pas révélé de différence entre les souches, bien que trois d'entre elles isolées en Inde et au Pakistan ont pu être distinguées des autres après digestion enzymatique par *Hpa*II des produits d'amplifiés par H3/H4. Mise à part ces quelques différences, il a été démontré que tous les adénovirus isolés de cas d'hydropéricardite infectieuse observés dans plusieurs pays appartenaient tous à l'adénovirus de sérotype 4.

ZUSAMMENFASSUNG

Epidemiologische Studien über Hühneradenovirusisolate aus Fällen von infektiösem Hydroperikard

Serologie, Restriktionsenzymanalyse und Polymerase-Kettenreaktionen wurden verwendet, um insgesamt 12 Isolate des Hühner-

adenovirus (FAV) zu klassifizieren, die aus Feldausbrüchen mit klinischen Symptomen von infektiösem Hydroperikard in 7 Ländern in Asien und Amerika stammten. Alle Isolate gehörten zum FAV-Serotyp 4. Zwei Isolate waren mit aviärem Adeno-assoziiertem Virus kontaminiert, und eins von ihnen enthielt außere Unterschiede wurden bei den *Sma*I-, *Bgl*II- und *Pst*I-Schnittmustern festgestellt. In den fünf verschiedenen *Pst*I-Schnittmustern waren jedoch über 80% der Fragmente in der Größe identisch, was auf die enge Genomverwandschaft zwischen den Isolaten hindeutet. Untersuchungen mit der Polymerase-Kettenreaktion bestätigten die Klassifizierung der Isolate als FAV4-Stämme. Alle Isolate konnten mit Hilfe der Primerpaare H1/H2 oder H3/H4 nachgewiesen werden. Die Restriktionsenzymanalyse der H1/H2-PCR-Produkte ermöglichte keine Differenzierung zwischen den Isolaten, während nach *Hpa*II-Spaltung der H3/H4-Produkte die drei Isolate aus Indien und Pakistan von allen anderen Isolaten unterschieden werden konnten. Obwohl Stamm-Variationen nachgewiesen wurden, konnte gezeigt werden, dass alle Adenoviren, die aus verschiedenen Fällen von infektiösem Hydroperikard (Angara Disease) in verschiedenen Ländern isoliert wurden, zum Hühneradenovirus-Serotyp 4 gehören.

RESUMEN

Estudios epidemiologicos en adenovirus aviares aislados en casos de hidropericardio infeccioso

Se utilizaron técnicas serológicas, de enzimas de restricción y de reacción de la polimerasa en cadena para la clasificación de un total de 12 adenovirus aviares (FAV), aislados en casos clínicos de hidropericardio infeccioso (IHP) y procedentes de casos de campo de 7 países de Asia y América. Todos los aislados pertenecían al serotipo 4 del FAV. Dos aislados estaban contaminados con virus asociados a adenovirus y uno también contenía FAV1. Se observaron escasas diferencias en los perfiles de restricción correspondientes a *Bam*HI. En los perfiles de restricción correspondientes *Sma*I, *Bgl*II y *Pst*I se detectó una mayor variabilidad. Sin embargo más del 80% de los fragmentos de los cinco perfiles *Pst*I presentaban un tamaño idéntico, lo que indica una estrecha relación genómica entre los aislados. Las técnicas de polimerasa en cadena confirmaron la clasificación de los aislados como cepas FVA4. Se pudieron detectar todos los aislados utilizando los pares de primers H1/H2 o H3/H4. Realizando análisis con enzimas de restricción de los productos obtenidos mediante PCR con H1/H2 no se observaron diferencias entre los diferentes aislados, mientras que en los productos obtenidos con los primers H3/H4, los aislados de India y Pakistan se pudieron diferenciar de los otros, después de la digestión con *Hpa*II. Aunque se demostró una cierta variación entre las diferentes cepas, se pudo evidenciar que todos los adenovirus aislados de varios casos de campo de hidropericardio infeccioso (Enfermedad de Angara) en diferentes países, pertenecen al serotipo 4 del adenovirus aviar.