Pathogenicity and Gene Analysis of Adenovirus from Pigeons with Inclusion Body Hepatitis

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ABSTRACT. The pathogenicity of an adenovirus isolated from pigeons (Pigeon adenovirus, PA) with inclusion body hepatitis in Taiwan was investigated in specific-pathogen-free (SPF) chicks and in racing pigeons. One-day-old SPF chicks were inoculated subcutaneously with 10^5 50% tissue culture infective dose (TCID_{50}, low dose) and 10^6 TCID_{50} (high dose) of the virus, respectively. The chicks began to die three days post inoculation (DPI) with high dose of the virus and the mortality reached 100%; the chicks began to die 6 DPI and the mortality reached 90% at 14 DPI with low dose. The adult pigeons seemed to be resistant to the PA. However, this virus decreased the production of antibody against Newcastle disease virus in pigeons. It is found that this PA belongs to genetic group D from the restriction patterns produced by BamHI and Hind III.

KEY WORDS: genotype, pathogenicity, pigeon adenovirus.

Adenoviruses are distributed worldwide. They are classified into two genera, Mastadenovirus and Aviadenovirus [3]. The aviadenoviruses can be divided into three main groups. The first group, group I, includes conventional adenoviruses, which share a common antigen. Group II contains viruses of hemorrhagic enteritis and group III contains viruses of egg-drop syndrome-76. Various avian species are infected with their own group I viruses [10]. Unlike the clear association of group II and group III adenoviruses with diseases, the role of group I avian adenoviruses as pathogens is not well defined. There are at least twelve serotypes of avian adenoviruses as can be seen by neutralization test [10]. Zsak and Kisary classified group I adenovirus into 5 genetic groups (from A to E) by restriction enzyme analysis [19]. Inclusion body hepatitis in pigeons is caused by a pigeon adenovirus (PA) and has been reported all over the world [2, 4, 5, 7, 9, 15, 17]. The disease is characterized by anorexia, polyuria, vomiting, and dehydration. Although the infection of this virus in pigeons is common in some countries, the role of PA in avian species is still unknown. This paper describes the characterization and pathogenicity of a PA isolate in Taiwan. The pathogenicity of this PA is low in adult pigeons but it induces high mortality in chicks. It belongs to genetic group D as defined by Zsak and Kisary [19] in group I avian adenovirus.

MATERIALS AND METHODS

Virus: A strain (1258/92) of pigeon adenovirus (PA) isolated from a carcass of a sick pigeon was used in the present study [17]. Three adult racing pigeons (Columba livia) from a pigeon loft with 30 birds showed signs of polydypsia, polyuria, anorexia, dehydration, and diarrhea after a long distance flying in September, 1992. The other 11 of them showed the same signs in 3 days (morbidity: 14/30). Altogether, four of them died in a week (mortality: 4/30). The virus was isolated by three passages in chicken embryo liver cell (CEL) and chick kidney cell cultures [17]. The PA in the supernatant was stained with 3% phosphotungstic acid and 0.01% bacitracin acid solution after centrifugation at 80,000 rpm (airfuge, rotor A-100, Babyman). The morphology of the virus was observed with an electron microscopy. The sixth passage of the virus isolated was plaque-purified three times. The purified virus was then propagated in SPF chick kidney cell in large quantity and concentrated by 10% (W/V) polyethylene glycol 6000 (Sigma) to reach 10^9 TCID_{50}. The identification and titration of the virus was conducted by indirect immunofluorescence (IF) with polyclonal anti-pigeon adenovirus antiserum (SPL-1 strain) [15].

Hemagglutination (HA) test: Red blood cells (RBC) were obtained from sheep, rats, mice, rabbits, chickens and geese and washed three times in phosphate-buffered saline (PBS). They were then suspended in PBS at two concentrations: 0.5% and 1%. Equal portions of virus (10^4 TCID_{50}) or control fluids were mixed together with the RBC suspension. The micro-plate was shaken for 5 min and observed for agglutination of the RBC's one hr later at 4°C, 25°C and 37°C. EDS76 virus Co strain (Animal Health Research Institute, Tamsui, Taiwan) was used as a positive control.

Pathogenicity in pigeons: Twenty-one adult pigeons (Columba livia, 6 months to one year of age) free from anti-PA antibody by neutralization test were divided into 3 groups; 9 in the low dose (10^4 TCID_{50}), 9 in the high dose (10^6 TCID_{50}) and 3 in the control (un-inoculated) group. The inoculated pigeons were housed in separate rooms and observed for two weeks before sacrifice.

Pathogenicity in day-old chicks: Thirty 1-day-old specific pathogen-free White Leghorn chickens (Animal Health Research Institute, Chi-Ding Branch, Chi-Ding, Taiwan) were used to determine the virulence of the isolate. Twenty
serum samples of them collected from the jugular vein of the chicks before inoculation were found negative to PA by neutralization test [16]. They were divided into three groups and housed in separate rooms of 10 birds in each group. 10³ TCID₉₀ and 10⁵ TCID₉₀ of virus were inoculated subcutaneously. Ten un-inoculated chicks were used as the control group.

Influence of PA on the production of anti-ND antibody in pigeons: Twenty adult pigeons free from the antibodies against PA and Newcastle disease virus (NDV) were divided into 4 groups. Four pigeons in group I as a control group, four in group II were inoculated intravenously with PA (10³ TCID₉₀) only, six in group III were inoculated intravenously with NDV (strain Ck/Tw/970/91, 10³-⁵ TCID₉₀) [8] only and another six in group IV were inoculated with PA and NDV simultaneously. The blood of the pigeons was collected before inoculation and 1, 2, 3, 4 and 5 weeks after inoculation and tested for the titers of anti-NDV by hemagglutination inhibition (HI) test and anti-PA antibody by neutralization test. The mean anti-NDV HI log₂ titer in pigeons inoculated with both NDV and PA was compared with the pigeons inoculated with NDV alone by a two-sample t test for the two-tailed hypothesis at 95% significant level [18].

Preparation of adenovirus DNA from infected cells: Viral DNA was extracted according to the method described by Shinagawa et al. [14]. Briefly, the infected cells were lysed with 1% SDS, 10 mM Tris-HCl and 10 mM EDTA, pH 7.5. Most cellular DNA was removed by adding 5 M NaCl. The viral DNA was separated from RNA and fragmented cell DNA by phenol extraction, and the viral DNA in the phenol layer and the interphase was precipitated by pure ethanol. The precipitate was digested with proteinase K (Gibco BRL), extracted by phenol and dissolved in distilled water.

Restriction enzyme analysis: One to two µg viral DNA were digested with Hind III, BamH I, Sal I, EcoR I, Cla I, Kpu I, Pst I, Xho I, and Sma I (New England Biolabs) according to the manufacturers specifications. Resulted fragments were separated in 0.8% agarose in TAE buffer (Amresco, U.S.A.) at 35 volts. High molecular weight and 1 kb plus DNA markers (Gibco, BRL) were included on each gel as DNA size standards.

Terminal fragment determination: Bal-31 exonuclease digestion was used to determine the terminal restriction enzyme fragments according to the method described by Pallister and Sheppard [11]. Ten µg of DNA were digested with Bal-31 exonuclease (New England Biolabs, U.S.A.). Nine µl of aliquots of the reaction mixture were removed at 0, 2, 4, 10, 20, and 30 min after addition of Bal-31 exonuclease and added to 1 µl of 200 mM ethylen-bis(oxy-ethylenenitrile) tetraacetic acid (Sigma, U.S.A.) to inhibit the enzyme activity. Each aliquot was digested with NdeI and AseI, respectively and then electrophoresed on a 0.8% agarose gel to determine the restriction digestion.

RESULTS

Growth of the pigeon adenovirus (PA): The pigeon adenovirus showed the typical morphology of adenovirus under electron microscopic examination (Fig. 1) and induced typical inclusion bodies in chicken kidney cells one day after inoculation. The virus in cell culture could be attached by the antisera prepared with a Japanese PA strain (SPL-1) with IF test. The ninth passage PA reached a plateau titer (10⁴ TCID₉₀) three days after inoculation. When the virus was

Fig. 1. Electron micrograph of pigeon adenovirus isolated from pigeon with inclusion body hepatitis. Bar: 100 nm.
harvested and concentrated by 10% (W/V) polyethylene glycol 6000 (Sigma), it reached 10^6 TCID_{50}.

**Hemagglutination of PA:** Agglutination of the six types of RBC's could not be observed in the plate HA tests at the tested temperatures. In contrary, EDS76 virus showed positive reaction in HA test.

**Pathogenicity in pigeons:** All the inoculated pigeons with low or high doses of PA showed no clinical sign till the end of the experiment. In microscopy, there was cyst formation in the bursa of Fabricius in one pigeon and lymphocyte depletion in the spleen of the other pigeon. However, their thymus showed no microscopical lesions.

**Pathogenicity in day-old chicks:** In subcutaneous injection groups, the chicks inoculated with 10^3 TCID_{50} showed signs of depression, anorexia, weakness and died in 3 days post inoculation (DPI). The mortality reached 100%. At the dose of 10^4 TCID_{50} virus, the inoculated chicks died in 6 DPI. The mortality reached 90% at 14 DPI. The affected birds showed lymphocytic depletion in the bursa, focal necrosis in liver and pancreas. Some basophilic intranuclear inclusion bodies were recognized in liver, and pancreas. No clinical signs and pathologic lesions were observed in the tissues of control chicks.

**Influence of PA on the production of anti-ND antibody in pigeons:** The anti-NDV antibody in pigeons after inoculation with PA and/or NDV is shown in Fig. 2. The HI titers in the pigeons in control group and the PA-inoculated group were negative (less than 2 log_2). The pigeons inoculated with NDV showed seroconversion one week after inoculation, reached a plateau three weeks later and then decreased after four and five weeks inoculation. The pigeons inoculated with both PA and NDV showed the same pattern as the pigeons inoculated with NDV alone but had slower and lower seroconversion than the latter (p<0.05). In other words, the PA decreased the production of anti-NDV HI antibody in pigeons.

**Restriction enzyme analysis:** Twenty to forty µg of viral DNA could be obtained from a 175 cm^2 tissue culture flask in DNA extraction. The restriction patterns of the PA DNA cleaved by restriction endonucleases, Hind III, BamH I, Sal I, EcoR I, Cla I, Kpn I, Pst I, Xho I, and Sma I were shown in Fig. 3. The DNA fragments ranging in size from 0.6 to 25.9 kb. Considering the fragments produced by BamH I and Hind III, this PA belonged to genetic group D as the grouping results described by Zsak and Kisary [19]. The genomic content of PA was found to be 45.6–48.2 kb as estimated by restriction enzymes digestion. This value is slightly longer than that of CELO (43.804 bp in length) [1].

**Terminal fragment determination using Bal-31 exonuclease:** Bal-31 exonuclease digestion of pigeon adenovirus showed that the Nde I 7.3 kb and 2.9 kb fragments were digested at the same time (Fig.4). The 2.9 kb fragment was digested completely. Using the same method, the Ase I 21.5 kb and 0.75 kb fragments and the Sfi I 28.3 kb and 19.9 kb were found to be the terminal fragments.

**DISCUSSION**

Inclusion body hepatitis of pigeons induced by herpesvirus was reported by several researchers [9, 13]. However, adenovirus should be added to the list of differential diagnosis of liver disease in pigeons when intranuclear inclusion bodies are present. Takase et al. [15] isolated a PA serotype 2 from pigeons with inclusion body hepatitis. Serotype 2 belongs to genetic group D as in Zsak’s classification [19].
Fig. 4. Digestion of PA using Bal-31 exonuclease. Samples were taken 0, 10, 20, and 30 min (lanes 1, 2, 3, and 4) after exonuclease digestion and DNA from each time point was then digested with Nde I. M1: high molecular weight DNA marker, M2: 1 kb plus DNA ladder.

Since both viruses belong to Group I avian adenovirus, which share the same group specific antigen this PA in cell culture could be caught by the antiserum specific to the Japanese isolate (SPL-1). Whether both viruses belong to the same serotype, needs further study. The pathogenicity of the PA was low to pigeons, which corresponds to Pallister and Sheppard’s report, in which, mild and hypervirulent fowl adenovirus belonged to genetic group E [11].

Adenoviruses do not usually produce disease by themselves; rather, that they are likely to be part of a multiple etiology for illness that also includes stress and immunosuppression [10, 12]. In the present study, immunosuppressive effect of PA was found in pigeons inoculated with NDV. This result corresponds to the pathological lesions found in the bursa of Fabricius and spleen but not in thymus. In Taiwan, sixty-three percent of pigeon lots were contaminated by PA and only a few of them showed clinical outbreak [16]. The severity of the clinical signs observed in a particular outbreak of PA infection depends on the pathogenicity of the infecting strain of virus as well as the presence of other agents or factors which may lower the individual’s ability to control the infection.

The role of PA in ND occurrence in pigeons is very important. Hoffmann et al. [6] suggested that infection with some strains of fowl adenovirus causes lymphoid depletion of bursa, thymus and spleen which may have an effect on both humoral and cell-mediated immunity. The bursal lesion in inoculated pigeons in the present study might be accounted for the delayed anti-ND antibody formation. The findings from this study support the hypothesis that an adenovirus alone does compromise the immune capacity of the host.

Extensive hepatic necrosis affecting pigeons of all ages was described recently by De Herdt et al. [2]. The large number of inclusion bodies in the liver of the infected pigeons clinically was certainly induced by adenovirus [17]. However, the present PA was not virulent to pigeons. It needs some stress factors to increase the susceptibility of pigeons, and over-doses or otherwise inappropriate use of anthelmintics and antibiotics could be stressful and could have exacerbated the adenovirus infection in pigeons. Despite the lower pathogenicity of PA in pigeons, it is found higher in SPF chicks. In addition, this PA grew well in chicken origin cells, the role of the PA in chicken industry needs further investigation.

REFERENCES


