

Epidemic Keratoconjunctivitis Caused by a New Genotype of Adenovirus Type 8 (Ad8)—A Chronological Review of Ad8 in Southern Taiwan

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Purpose: To understand the pathogenic evolution of viral keratoconjunctivitis in Kaohsiung, Taiwan, a retrospective molecular and clinical analysis was conducted.

Methods: From January 1990 to December 1994, conjunctival swab samples from patients suspected of having viral conjunctivitis were collected for viral culture isolation, neutralization test (NT), and endonuclease cleavage analysis. Six restriction endonucleases, comprising *HindIII*, *BamHI*, *SalI*, *SstI*, *SmaI*, and *PstI*, were used for cleavage. Clinical examinations of patients were performed by two senior ophthalmologists.

Results: Twenty-one cases of a new genotype of adenovirus (Ad) type 8, designated as Ad8H, were discovered in the 27 detected adenovirus cases. The Ad8H has a distinct cleavage pattern, especially by *HindIII* and *SalI*. The Ad8H keratoconjunctivitis induced more subconjunctival hemorrhage (33.3%), keratitis (33.3%), and lymphadenopathy (85.7%) than other genotypes of Ad8 previously isolated in Kaohsiung, Taiwan.

Conclusion: We have discovered a new genotype, Ad8H, which was prevalent as the main pathogen of the adenoviral keratoconjunctivitis in Kaohsiung, Taiwan from 1990 to 1994. Adenovirus type 8 is evolving into more genotypes with a trend towards more severe symptoms, including subconjunctival hemorrhage, keratitis, and lymphadenopathy. **Jpn J Ophthalmol** 2001;45:160–166 © 2001 Japanese Ophthalmological Society

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Introduction

Adenovirus is the main pathogen for viral keratoconjunctivitis. Adenovirus (Ad) types 3(Ad3), 4(Ad4), 7(Ad7), 8(Ad8), 11(Ad11), 19(Ad19), and 37(Ad37) may cause viral keratoconjunctivitis. Before 1990, Ad8 was the most commonly isolated adenovirus of viral keratoconjunctivitis^{1–5} in the Kaohsiung area of Taiwan. With advances in molecular biology, the use of endonuclease cleavage patterns further separated Ad8 into 7 genotypes^{1,3,4,6–8} comprising Ad8A, Ad8B, Ad8C, Ad8D, Ad8E, Ad8F,

and Ad8G. This series of studies was conducted in the 1980s by collaborative studies in Japan, Taiwan, and Korea.^{7,8} Other types of adenoviruses causing keratoconjunctivitis were also classified into several genotypes using the endonuclease cleavage pattern. Ad19 was classified into Ad19P, Ad19A^{9,10,11} and Ad19B,¹⁰ and Ad37 was classified into Ad37P, Ad37A,¹⁰ and Ad37B.¹⁰ Using different DNA restriction endonucleases, Noda et al¹² also classified Ad37 into a prototype Ad37p and 2 genotypes; Ad37a and Ad37b. Ad3, Ad4, and Ad11 were also classified into several genotypes.^{13–16}

The laboratory diagnosis of viral conjunctivitis by culture isolation and neutralization test (NT) usually takes 1 to 3 weeks, while diagnosis by polymerase chain reaction (PCR)^{17–19} or PCR and restriction

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fragment length polymorphism (PCR-RFLP)²⁰ takes less than 3 days. Although all the PCR methods are quick for diagnosing adenoviral infection, they may not identify genotypes of adenovirus. In this study, we performed a nested PCR and RFLP, as described by Saito-Inagawa et al.²⁰, in order to test if by using PCR-RFLP it is possible to differentiate the genotypes of Ad8. The nested PCR can amplify a 956-bp DNA fragment of the conserved region in hexon genes of 14 types of Ad. Furthermore, with three restriction enzymes, *EcoT14I*, *HaeIII*, and *HinfI*, an RFLP analysis can detect different types of adenovirus. We applied this PCR-RFLP method directly to the previously extracted DNA of all Ad8 genotypes.

In this study, we tried to identify the viral pathogen of keratoconjunctivitis in Kaohsiung, Taiwan from 1990 to 1994 using culture isolation and NT. To distinguish the genotypes of the isolated adenoviruses, we used endonuclease cleavage patterns by 6 restriction endonucleases: *PstI*, *BamHI*, *HindIII*, *SallI*, *SstI*, and *SmaI*.

There were two epidemics of acute hemorrhagic conjunctivitis (AHC) in Taiwan during this period, one in 1990 and the other in 1994. To rule out conjunctivitis infected by enterovirus type 70 (EV70) and coxsackievirus A type 24 variant (CA24v), we did reverse transcription-polymerase chain reaction (RT-PCR) as described by Lin et al.²¹ to detect EV70 and CA24v in all our samples.

Materials and Methods

Specimen Collection

From January 1990 to September 1994, patients suspected of having viral conjunctivitis were examined thoroughly by two senior ophthalmologists (Dr. M. M. Sheu and Dr. W. L. Huang) in the Department of Ophthalmology, Kaohsiung Medical University Hospital. The conjunctival swabs collected in 3-mL viral transport media were sent immediately to the viral laboratory at Kaohsiung Medical University in iceboxes for analysis.

Culture Isolation and NT

Viral cultures were established using HeLa cells and A549 cells. The viral cultures were examined for two passages, 7 days per passage, to observe cytopathic effects. If it was suspected that a cytopathic effect was caused by the adenovirus, the infected cells were identified by the immunofluorescence antibody technique using an Ad monoclonal antibody (Imagen™ Adenovirus test; DAKO, Denmark House, Angel Drove, Ely Cambs. CB7 4ET, United

Kingdom). Neutralization tests using anti-adenoviral sera were performed. All of the adenovirus isolates were propagated in A549 cells cultured in minimum essential medium supplemented with 2% fetal calf serum. If enterovirus-specific cytopathic effects were suspected, NT using anti-EV70 and anti-CA24v was done. If enterovirus-specific cytopathic effects were not found, RT-PCR for CA24v and EV70 was performed as described by Lin et al.²¹

Restriction Endonucleases and Cleavage Pattern Analysis of Isolated Adenoviruses

The DNA of viral infected cells was extracted essentially according to Lin et al.²² A549 cells in a 150-mL bottle were inoculated with adenovirus at a multiplicity of 1–10 plaque forming units (PFU/cell). When 95% of cytopathic results appeared, the infected cells were collected by centrifugation, washed with phosphate-buffered saline (PBS), and suspended in 4 mL buffer solution (pH 8.1) containing 0.05 M Tris-HCl and 0.01 M ethylenediaminetetraacetic acid disodium salt (EDTA). The infected cells were lysed and the digested by the addition of 1% sodium dodecyl sulfate and proteinase K (50 µg/mL) in a 65°C water bath for 1 hour. DNA was extracted twice with one volume of buffer-saturated phenol-chloroform-isoamyl alcohol (25:24:1) and two times with two volumes of chloroform. The extracted DNA was vacuumed and heated at 65°C for 15 minutes to remove trace amounts of chloroform in the aqueous phase. The samples were then treated with RNase (10 µg/mL) at 65°C for 40 minutes. Thereafter, DNA was precipitated with two volumes of cold ethanol, dried by vacuum and dissolved in 1 mL 0.01 M Tris-HCl and 0.001 M EDTA. Four micrograms of extracted DNA was subjected to 25 U each of 6 restriction endonucleases, *PstI*, *BamHI*, *HindIII*, *SallI*, *SstI* and *SmaI*, in a 40-µL reaction solution, as described by Fujii et al.⁶ The digested products were applied on a 1% agarose gel electrophoresis (50 V) in TBE (Tris-Borate) solution for 13 hours. After electrophoresis, the gel stained with ethidium bromide was visualized and photographed under ultraviolet light.

Using PCR-RFLP to Identify Genotypes of Ad8

The previously extracted DNA from 7 genotypes of Ad8, Ad8A through Ad8G, was subjected to PCR-RFLP by Saito-Inagawa's method.²⁰ In the nested PCR, the primers for the first PCR were AdTU7 (GC-CACCTTCTTCCCCATGGC, nucleotide numbers 20,734-20,753) and AdTU4' (GTAGCGTTGCCG-GCCGAGAA, nucleotide numbers 21,718-21,737),

and the primers for the second PCR were AdnU-S' (TTCCCCATGGCNCACAACAC, nucleotide numbers 20,743-20,762) and AdnU-A (GCCTCGATGACGCCGCGGTG, nucleotide numbers 21,679-21,698).

The first PCR was performed with extracted DNA in a 50- μ L volume containing 20 pmole of each primer, 1 mM of each deoxynucleotide triphosphate (ie, dATP, dGTP, dCTP, dTTP), 1 U of thermostable *Taq* DNA polymerase, 5 mM Tris-HCl, 0.01 M NaCl, 0.025 mM EDTA, 0.1 mM 2-mercaptoethanol, and 5% glycerol (V/V). PCR was carried out in a thermal cycler (Hybaid, Middlesex, UK) by using a cycle of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute, and primer extension at 72°C for 2 minutes, for 36 cycles. The nested-PCR amplification was performed with 5 μ L of the first PCR product in a 50- μ L volume by using the same protocol as with the first PCR. After the nested-PCR amplification, 5 μ L of the reaction mixture was subjected to electrophoresis on a 1% agarose gel containing 0.5 μ g ethidium bromide per mL. The secondary PCR products were digested separately by three restriction endonucleases, *Eco*T14I, *Hae*III, and *Hinf*I, and studied electrophoretically on a 3% agarose gel containing 0.5 μ g ethidium bromide per mL. After photographing, the DNA restriction fragments were analyzed.

Results

Laboratory Diagnosis

From January 1990 to December 1994, 339 conjunctival swab samples were collected. One hundred and forty-six of the samples were diagnosed as CA24v infections by culture isolation, NT and RT-PCR. Twenty-seven samples were detected as Ad infection by culture isolation and the immunofluorescence antibody technique using an Ad monoclonal antibody. NT results showed that 21 samples were Ad8, two samples Ad3, two samples Ad11, and two samples Ad37. Further restriction endonucleases cleavage pattern analysis revealed that the 21 isolates of Ad8 had an identical new cleavage pattern, which was different from our previous findings (Figure 1). They have distinct patterns when cleaved by *Hind*III and *Sal*I. We designated this new type as Ad8H in accordance with the serial studies in which Japan and Korea collaborated, as mentioned previously. The two isolates of Ad3 (Figure 2) and the two isolates of Ad11 (Figure 3) also had cleavage patterns different from what we have seen before. We designated these new genotypes as Ad3C and

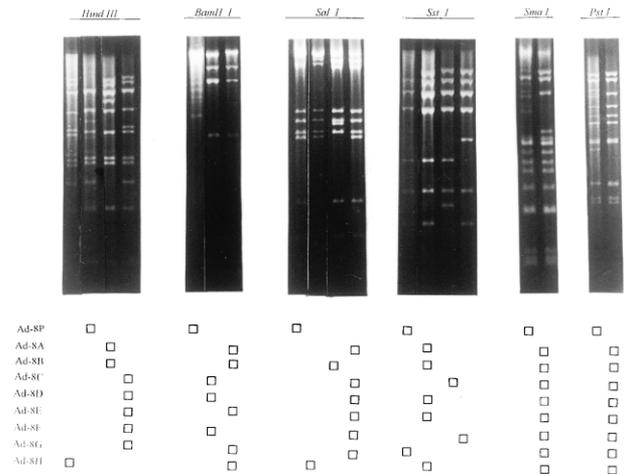


Figure 1. Cleavage patterns of adenovirus type 8 (Ad-8) with six restriction endonucleases: The open square (□) indicates cleavage pattern of Ad-8 genotype identical to the pattern of that lane. Ad-8P: Strain Trim.

Ad11B. Another two isolates of Ad37 were identified as Ad37P.

Clinical Analysis of Ad8

Keratoconjunctivitis Cases

The ages of these 21 cases with Ad8H keratoconjunctivitis ranged from 2 to 54 years, with a mean of 32.4 years. The sex ratio was 15 males to 6 females. All 21 cases (100%) had follicles and conjunctivitis (Table 1). Seven cases (33.3%) showed subconjunctival hemorrhage. These subconjunctival hemorrhage patterns were grossly classified as extravasation, petechia, and large blots or patches of hemorrhage. Extravasation and petechia existed in both palpebral

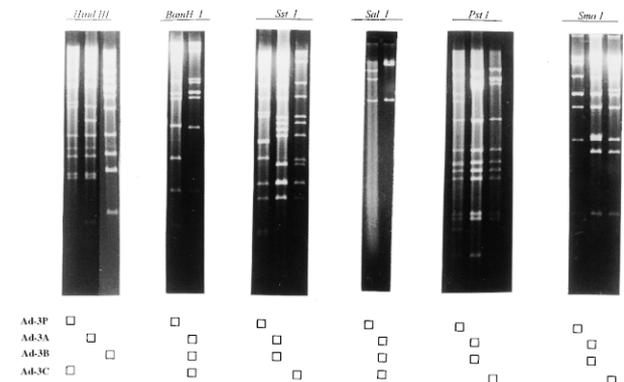


Figure 2. Cleavage patterns of adenovirus type 3 (Ad-3) with six restriction endonucleases: The open square (□) indicates cleavage pattern of Ad-3 genotype identical to the pattern of that lane.

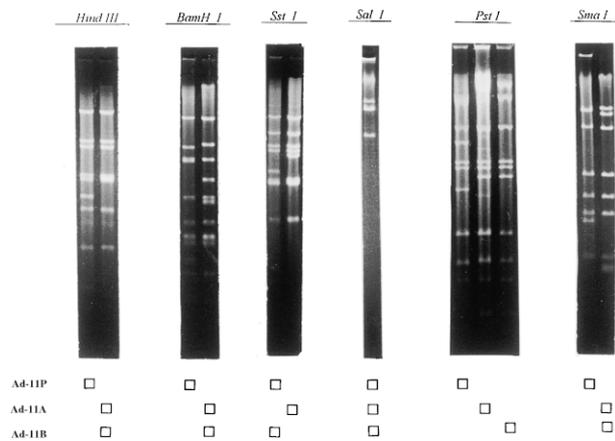


Figure 3. Cleavage patterns of adenovirus type 11 (Ad-11) with six restriction endonucleases: The open square (□) indicates cleavage pattern of Ad-11 genotype identical to the pattern of that lane.

and bulbar conjunctiva cases. Large blots or patches of hemorrhage occurred in bulbar conjunctiva cases. Three of the 7 patients with these patterns had large blots and patches of hemorrhage. Seven cases (33.3%) had keratitis. The keratitis included diffuse superficial epithelial keratitis, focal epithelial lesions, and subepithelial opacities. Six of the 7 patients with keratitis had diffuse superficial epithelial keratitis. Eighteen (85.7%) cases were positive for preauricular lymphadenopathy, 7 of which showed tenderness. Ten cases (47.6%) had extraocular symptoms, including sore throat, nasal discharge, fever, and nausea. Other eye symptoms were as follows: 14 cases (66.6%) with eyelid swelling, 10 cases (47.6%) with chemosis, 21 cases (100%) with eye discharge, and 19 cases with lacrimation (80.9%). Eleven cases (52.4%) had both eyes infected on examination.

Table 1. Symptoms and Signs in 21 Detected Patients with Adenovirus Type 8H Keratoconjunctivitis

Symptoms and Signs	No. of Patients*
Follicles	21 (100)
Conjunctivitis	21 (100)
Subconjunctival hemorrhage	7 (33.3)
Keratitis	7 (33.3)
Lymphadenopathy	18 (85.7) [†]
Extraocular symptoms	10 (47.6)
Lid swelling	14 (66.6)
Chemosis	10 (47.6)
Eye discharge	21 (100)
Lacrimation	17 (80.9)
Bilateral involvement	11 (52.4)

*Values in parentheses are percentages.

[†]Seven cases with tenderness.

Genotype Analysis of Ad8 Using PCR-RFLP

Although all seven genotypes had positive secondary PCR products, RFLP of the secondary PCR products by the three restriction enzymes showed little variation among these 7 genotypes (Figure 2). The seven genotypes of Ad8, Ad8A to Ad8G, could not be distinguished from each other by this method.

Discussion

Since the first isolation of Ad8 as an etiology of epidemic keratoconjunctivitis (EKC) in 1955 by Jawetz et al,^{23,24} many Ad8 genotypes have been identified after the application of restriction endonucleases and cleavage pattern analysis. Seven genotypes of Ad8 have been discovered in Sapporo, Busan, and Kaohsiung.^{1,3,4-7} Ad8A and Ad8B were first isolated in Sapporo by Fujii et al.⁶ Ad8C, Ad8D, Ad8E, Ad8F, and Ad8G were detected in Kaohsiung from 1980 to 1987.^{1,3,4,8} Ad8C was most prevalent from 1980 to 1981⁸ and Ad8E was most prevalent from 1983 to 1987.⁸ In this study, a new type of Ad8, Ad8H, was found to be the exclusively prevalent pathogen of Ad8 in Kaohsiung from 1990 to 1994.

In comparison with the clinical pictures of EKC caused by Ad8C and Ad8E during the 1980s in Kaohsiung as shown in the study by Sheu et al,⁸ more keratoconjunctivitis with subconjunctival hemorrhage, keratitis, and preauricular adenopathy (Table 2) have been noticed in the Ad8H infection in this series. Although there were more extraocular symptoms than in our previous experience with Ad8,² we would not regard them as secondary illnesses to Ad8. We might have a clearer picture if we had done the viral culture from patients' throat swabs and stools. In general, there is a trend towards

Table 2. A Comparison of Clinical Features of Adenovirus Type 8 (Ad8) C, Ad8E, and Ad8H

Clinical Features*	Ad8C	Ad8E	Ad8H
	(23 Cases) (Reference 8)	(41 Cases) (Reference 8)	(21 Cases) (This study)
Prevalence years	1980-1981	1983-1987	1990-1994
% SCH [†]	8.7	19.5	33.3
% Keratitis [‡]	13.0	12.2	33.3
% PAL ^{†‡§}	34.8	41.5	85.7

*SCH: Subconjunctival hemorrhage, PAL: preauricular lymphadenopathy.

Chi-square tests:

[†]Ad8C and Ad8H significantly different ($P < .05$).

[‡]Ad8E and Ad8H significantly different ($P < .05$).

[§]Ad8C, Ad8E and Ad8H significantly different ($P < .05$).

more serious symptoms and signs for Ad8H keratoconjunctivitis than for Ad8C.

Ad19 and Ad37 have become common pathogens of EKC since 1973,¹¹ and a mixed infection with Ad8, Ad19, and Ad37 in the epidemics of EKC has been noticed frequently.^{25,26} New genotypes of Ad19¹⁰ and Ad37¹⁰ in the Kaohsiung area were detected in 1983 and 1984. New genotypes of Ad8 evolved at an even faster rate than Ad19 and Ad37 with more than eight genotypes discovered. As reported by de Jong et al,²⁷ in four consecutive epidemics of Ad8 keratoconjunctivitis in France from 1983 to 1988, Ad8 also displayed considerable variability. Because the restriction enzymes they used were different from what we used, it is impossible to compare our genotypes with theirs. However, Kemp and Hierholzer²⁸ reported that Ad keratoconjunctivitis was consistently caused by the prototype of Ad8 (Trim type) in geographically separated populations, including the United States, Athens (Greece), and Taipei (Taiwan). They identified 17 out of 25 samples in the early 1980s to be Ad8 prototype from Taipei, which is located in northern Taiwan. But in southern Taiwan, Kaohsiung, Sheu et al⁸ had found a great variation of Ad8 as a keratoconjunctivitis pathogen from 1980 to 1987. In our present study, Ad8 in southern Taiwan was found to have further evolved in the early 1990s.

Why Ad8 evolved continuously and induced more serious cases of keratoconjunctivitis deserves further investigation. In Kaohsiung, although adenoviral keratoconjunctivitis tends to occur in the warm season, there are sporadic cases the whole year round.^{1,2,8,10} Probably because of the frequency of infection in the population, Ad8 evolved diversely in the Kaohsiung area. Another reason for the diverse evolution of Ad8 in this area may be due to the frequent use of steroid eye medication to treat EKC. The host's immune suppression by steroids might induce a longer course of infection.^{29,30} The longer presence of Ad8 in host cells might result in further evolution of Ad8 as described for CA24v.³¹ The evolution of Ad8 has resulted in more serious symptoms of keratoconjunctivitis, such as subconjunctival hemorrhage, keratitis, and preauricular lymphadenopathy. Whether this change is caused by the increased virulence of Ad8 or another mechanism such as antibody-enhanced activation by previous infection³² is a topic for further research.

Polymerase chain reaction and PCR-RFLP have been recognized as quick methods to diagnose viral infection of keratoconjunctivitis.¹⁹ For typing of adenoviruses, PCR-RFLP as described by Saito-Ina-

gawa et al²⁰ has successfully differentiated 14 prototypes. But as the adenoviruses evolve, the main causative pathogens of EKC or pharyngoconjunctival fever are not always prototypes. For example, Ad19A is more common than Ad19P in causing EKC. Adenovirus type 8 had several genotypes as the dominant pathogens for EKC in different areas. So, although PCR and PCR-RFLP are quick methods for diagnosis with high sensitivity, it is necessary to know if they make it possible to differentiate between genotypes of adenoviruses. In this study, we have tried to differentiate seven genotypes of Ad8, but the result was not satisfactory. For a complete epidemiological study to reveal the genotypes and the evolution of Ad, the endonuclease cleavage pattern analysis of the isolated virus is indispensable. An easier and faster method to classify Ad8 genotypes needs to be developed in the future.

A new genotype of Ad8 evolved in the Kaohsiung area and established itself as the main viral pathogen

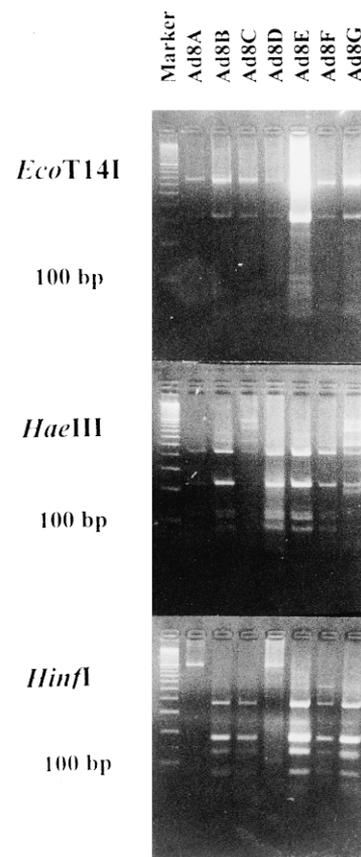


Figure 4. Polymerase chain reaction and restriction fragment length polymorphism of seven genotypes of adenovirus type 8 (Ad 8). The seven genotypes of Ad8 cannot be distinguished by the three restriction endonucleases: *EcoT14I*, *HaeIII*, and *HinfI*. Marker: 100 base pair ladder.

of adenoviral keratoconjunctivitis from 1990 to 1994. This new type of Ad8 was designated as Ad8H in accordance with a series of collaborative studies in the Far East,⁷ including Japan, Taiwan, and Korea. The Ad8H presented itself with worse symptoms and signs, such as subconjunctival hemorrhage, keratitis, and preauricular lymphadenopathy, than Ad8C and Ad8E. A further follow-up of Ad8 in Taiwan and the Far East is important for monitoring its evolution. An answer to the question of why Ad8 evolved at such a steady rate in Kaohsiung requires an epidemiological study in this area. A dense population, humid and warm weather, and unrestricted use and availability of steroid eye medication may be factors worth investigating. In addition, the development of a quick and easy method to detect the genotype of adenovirus causing keratoconjunctivitis would be helpful in future epidemiological study.

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