Jpn. J. Infect. Dis., 65, 260-263, 2012

Short Communication

A Molecular Epidemiologic Study of Human Adenovirus Type 8 Isolates Causing Epidemic Keratoconjunctivitis in Kawasaki City, Japan in 2011

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(Received December 1, 2011. Accepted March 16, 2012)

SUMMARY: A local outbreak of epidemic keratoconjunctivitis (EKC) caused by human adenovirus type 8 (HAdV-D8) occurred in Kawasaki city, Japan in July–August 2011. Since the cases were sporadic in nature, the source of the infection could not be identified. The results of PCR analysis and the appearance of cytopathic effects in the samples indicated that 22 patients were positive for HAdV. The mean age of the patients (10 men and 12 women) was 64.3 ± 17.3 years (median, 68 years; range, 11–86 years). The sequences of hexon, which included hypervariable loop 1; the penton, which included RGD loops; and the fiber, which included the knob-coding regions, were identical in all the HAdV-positive cases. Phylogenetic analysis of the major capsid protein-encoding regions of HAdV confirmed that the isolates were HAdV-D8. Although the incidence of HAdV-D8 outbreaks has decreased in Japan since 1997, the results of our study imply that HAdV-D8 is still a causative agent for EKC outbreaks in Japan.

A total of 22 outpatients (10 men and 12 women) were diagnosed with epidemic keratoconjunctivitis (EKC) at a hospital in Kawasaki city, Japan during routine national surveillance from July 19 to August 17, 2011 in Japan. The mean age of the patients was 64.3 ± 17.3 years (median, 68 years; range, 11–86 years). The EKC outbreak occurred in a limited area (Kawasaki ward) of the city. It was difficult to identify the source of infections since the cases were sporadic in nature. However, we observed that in 4 (14%) cases, the infection were familial. Conjunctival swabs of the patients were collected after 3.0 ± 2.5 days (mean ± S.D.) from the onset of the disease.

All the samples (150 μL) were inoculated onto confluent monolayers of A549 cells (1). The inoculated cells were maintained in minimal essential medium supplemented with 1% fetal bovine serum (Cansera International Inc., Toronto, Canada). The cultures were observed for 3–4 weeks for the appearance of cytopathic effects (CPEs). After the cells showed clear CPEs, viral DNA was extracted from the supernatant of the infected cells using QIAamp viral RNA mini kit (Qiagen, Hilden, Germany). Human adenovirus (HAdV) strains were isolated from the cells of all the patients (n = 22). These isolates were typed using PCR sequencing and phylogenetic analysis of the regions encoding the structural proteins (hexon, fiber, and penton).

PCR reactions were performed in a 96-well Veriti thermalcycler (Applied Biosystems, Foster City, Calif., USA) with a total reaction volume of 50 μL, which contained 20 pmol/μL of each primer, 2.5 mM of dNTP, 1.25 U of GXL DNA polymerase (Takara, Shiga, Japan), and 5 μL of the DNA template. Thermal cycling was performed for 30 cycles; each cycle consisted of these following steps: denaturation at 98°C for 30 s, annealing at 55°C for 30 s, and elongation at 68°C for 2 min, followed by a final extension step at 68°C for 7 min. The following primers were used: hexon PCR primers, HX5-3 (forward 5′-CACATCGCCGGACA GGATGCTTCGGAGTA-3′) and HX3-4 (reverse 5′-GTGTTGTGAGCCATGGGGAAGAAGGTGGC-3′); hexon sequencing primers, S52 (reverse 5′-CCCATGTT GCCAGTGCTGTTGTARTACA-3′) (2) and Ad-hexNUF (forward 5′-AACAARTTYAGRAAYCCC AC-3′); penton PCR primers, Ad-penUF (forward 5′-CARAAYGAYCACAGCAACTT-3′) and Ad-penUR (reverse 5′-GCRGGMACGTTTTCACTRACGGT-3′); penton sequencing primers, Ad-penNUF (forward 5′-GGNTGCGGVGTDGAYTTYAC-3′) and Ad-penNUR (reverse 5′-CGRAARGTBACNGGRTCTTGCAT-3′); fiber PCR (and sequencing) primers, AdD1 (forward 5′-GATGTCAAATTCCTGGTCCAC-3′) and AdD2 (reverse 5′-TACCCGTGCTGGTGTAAAAATC-3′) (3). The PCR products were separated on a 3% agarose gel for 50 min at 100 V. DNA was purified from the PCR products using MinElute PCR purification kit (Qiagen) according to the manufacturer's recommendations. Cycle sequencing of gel-purified products was conducted using the Genome Lab DTCS Quick Start Kit (Beckman Coulter Inc., Fullerton, Calif., USA) with a total of 30 cycles at 96°C for 20 s, 50°C for 20 s, and 60°C for 2 min, and the products were analyzed on a CEQ 2000XL DNA Analysis System (Beckman Coulter). The

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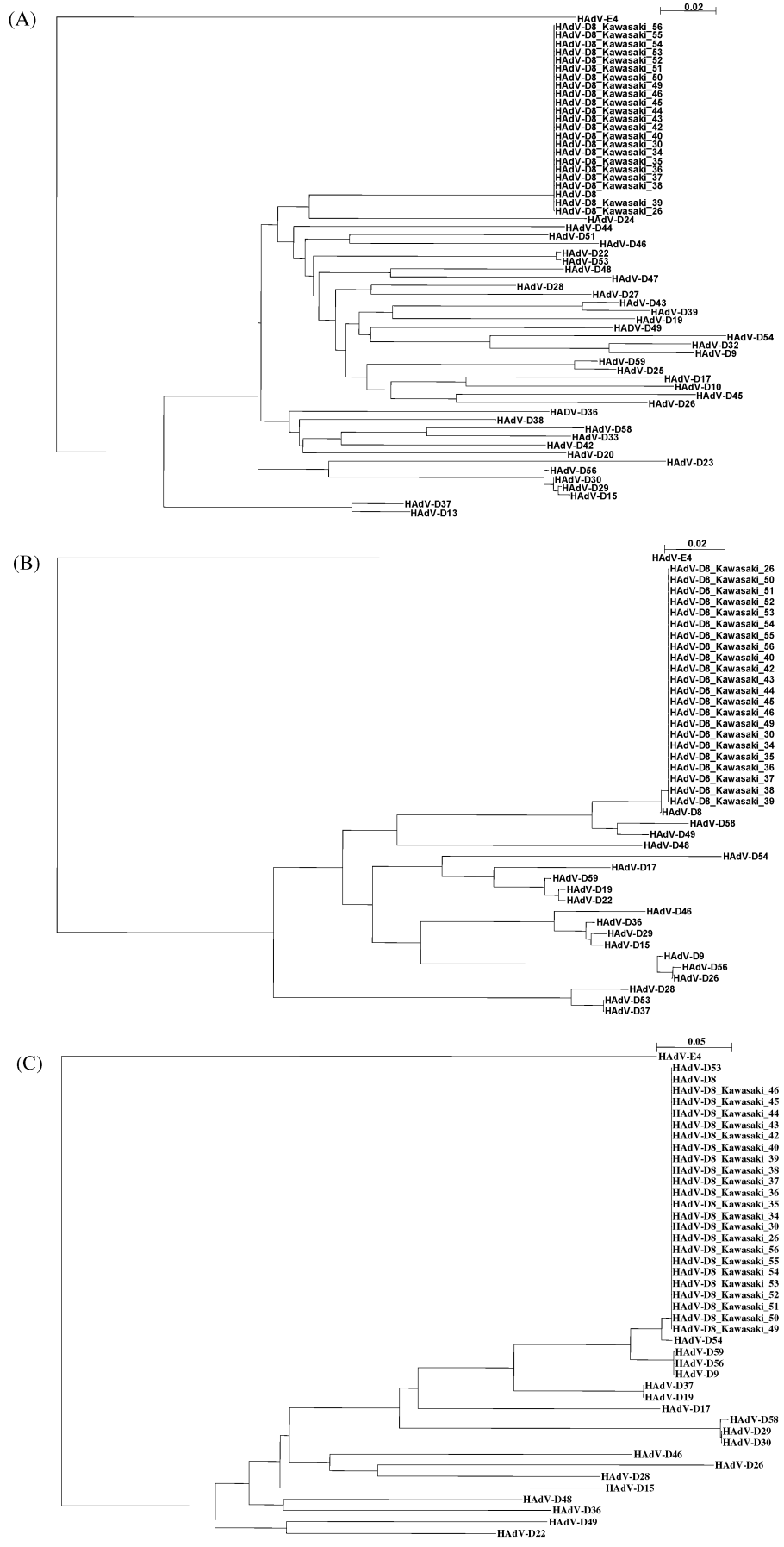

Fig. 1. Phylogenetic trees of HAdV-D8 positives and other HAdV-D reference strains in (A) the partial hexon gene (675 bp) include the loop 1, (B) partial penton gene (484 bp) including the RGD loop, and (C) partial fiber gene (410 bp) including the knob. The scale indicated nucleotide substitutions per position.

DNA sequences were assembled with CEQ 2000XL DNA Analysis System Software (version 4.3.9; Beckman Coulter). The DNA sequences were aligned using DNASIS Pro (version 3.0; Hitachi Solutions, Tokyo, Japan).

The data sets were aligned using CLUSTAL W (www.clustal.org), and phylogenetic trees were generated with 1,000 bootstrap resamplings using the neighbor-joining method. The genetic distance was calculated using Kimura's two-parameter method. The partial hexon, penton, and fiber sequences for the human adenovirus type 8 (HAdV-D8) strain (HAdV-D8_Kawasaki_26) were deposited in the DDBJ under GenBank accession numbers of AB685335, AB685336, and AB685337, respectively. The following HAdV genome sequences with GenBank accession numbers were used as HAdV-D reference sequences: HAdV-D8 (DQ149614, AB448767), HAdV-D9 (AJ854486), HAdV-D10 (DQ149615), HAdV-D13 (DQ149616), HAdV-D15 (DQ149617, AB562586), HAdV-D17 (AF108105), HAdV-D19 (DQ149618, EF121005), HAdV-D20 (DQ149619), HAdV-D22 (DQ149620, FJ404771), HAdV-D23 (DQ149621), HAdV-D24 (DQ149622), HAdV-D25 (DQ149623), HAdV-D26 (DQ140624, EF153474), HAdV-D27 (DQ149625), HAdV-D28 (DQ149626, FJ824826), HAdV-D29 (DQ149627, AB562587), HAdV-D30 (DQ149628, AF447393), HAdV-D32 (DQ149629), HAdV-D33 (DQ149630), HAdV-D36 (DQ149631, GQ384080), HAdV-D37 (DQ149632, DQ900900), HAdV-D38 (DQ149633), HAdV-D39 (DQ149634), HAdV-D42 (DQ149635), HAdV-D43 (DQ149636), HAdV-D44 (DQ149637), HAdV-D45 (DQ149638), HAdV-D46 (DQ149639, AY875648), HAdV-D47 (DQ149640), HAdV-D48 (EF153473), HAdV-D49 (DQ149641, DQ393829), HAdV-D51 (DQ149642), HAdV-D53 (FJ169625), HAdV-D54 (NC012959), HAdV-D56 (HM770721), HAdV-D58 (HQ883276), HAdV-D59 (JF799911), and HAdV-E4 (AY487947).

The partial hexon sequence, which included hypervariable loop 1; the penton sequence, which included the RGD loops; and the fiber sequence, which included the knob-coding region, were identical in all the cases. In phylogenetic analysis, all 22 isolates formed a monophyletic cluster with HAdV-D8 and were thus identified as HAdV-D8 (Fig. 1). Moreover, the HAdV-D8 isolates showed 99.9% nucleotide identity with the HAdV-D8 reference strain for the partial hexon sequence, 99.8% nucleotide identity with the HAdV-D8 reference strain for the partial penton sequence, and 100% nucleotide identity with the HAdV-D8 reference strain for the partial fiber sequence.

Most of the severe EKC infections are caused by HAdV species D. As reported previously (4), identification of HAdV species D on the basis of conventional serological tests alone leads to ambiguous results. In this study, PCR-sequencing methods with primers designed from the partial hexon (2), fiber (3), and penton genes (developed in this study) were used for accurate identification of HAdV. The HAdV-D8 strains detected in this study had no recombination events in the main capsids. The generations of novel recombinant HAdVs have been reported in recent studies. The novel HAdVs were created via multiple recombination events in the hexon, penton, and fiber genes. This indicates the importance of screening possible recombination events in these main capsid genes by performing sequence analysis.

We studied the complete nucleotide identities of all the HAdV-D8 isolates. These isolates were highly identical to the HAdV-D8 reference strain. These results suggest that the EKC outbreak was caused by the same HAdV-D8 strains but with a few mutations. Successive alterations were observed in the etiology of EKC worldwide between 1970 and 1980. In 1973, numerous epidemics of HAdV-D19 occurred, far exceeding the number of HAdV-D8 infections. Thereafter, HAdV-D37 was identified to be the infectious agent responsible for EKC epidemics throughout the world from 1976 to 1983 (5,6). In Japan, successive changes have been noted in the major types of EKC-causing HAdVs. The number of HAdV-D8 isolates was more than those of HAdV-D19 or HAdV-D37 from 1982 to 1996, except in 1985, 1986, and 1990, when HAdV-D37 was the dominant agent (7,8), and from 1997 to 1999, when HAdV-D19 was the predominant agent causing EKC (among the cases in which HAdV-D19 was detected [n = 325], 286 [82%] were diagnosed as EKC) (8). From 2000 to 2007, the number of HAdV-D37 isolates exceeded those of HAdV-D8 and D19, except from 2005 to 2006, when HAdV-D8 was dominant (9). According to previous reports (7–9), HAdV-D8 isolates comprised 4.88 ± 3.06% (mean ± S.D.; median, 3.65%; range, 1.47–10.5%) of the total number of reported HAdV isolates each year in the period between 1982 and 1996 in Japan. In contrast, the corresponding number in the period between 1997 and 2007 reduced to 1.45 ± 1.35% (mean ± S.D.; median, 1.17%; range, 0.35%–5.3%) of the reported number of HAdVs. The detection rates of HAdV-D8 in these 2 periods were significantly different when assessed by Mann-Whitney's U-test (P = 0.001).

Kaneko et al. (10) tested 115 HAdV isolates, which were identified as HAdV-D8, by neutralization test (NT). These strains were isolated from clinical samples collected in Japan between 1990 and 1999. The findings of their study showed that HAdV-D8 has become far less common since 1997 because only 1 and 3 strains were isolated in 2001 and 2003, respectively, and all the strains detected since 2004 were HAdV-D54 (not HAdV-D8). Our previous study, which was conducted using molecular methods between 2002 and 2010 in Fukui Prefecture, detected no HAdV-D8 and 64 HAdV54 strains in EKC positive cases (4). These results strongly suggest that HAdV-D8 strains detected (and identified by NT) after 2007 include falsely identified HAdV-D54 strains. Recent outbreaks of EKC were found to be caused by HAdV-D54 (11,12). Under these circumstances, it was essential to prove that the EKC outbreak in Japan after 2007 was caused by HAdV-D8.

In conclusion, HAdV-D8 is still present in the population of Japan and is a causative agent of EKC. However, the reason for this outbreak of HAdV-D8 EKC infections in elderly patients in Kawasaki city remains unidentified.

Acknowledgments We thank the hospital staff in Kawasaki city, Japan for the collection of conjunctival samples.

This study was partly supported by the Ministry of Health, Labour and Welfare of Japan (Research Promotion of Emerging and Re-

emerging Infectious Diseases).

Conflict of interest None to declare.

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