JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2001, p. 2975–2977 Vol. 39, No. 8
0095-1137/01/$04.00+0 DOI: 10.1128/JCM.39.8.2975–2977.2001

Identification of the Hexon Region of an Adenovirus Involved in a New Outbreak of Keratoconjunctivitis

YASUO IMAI, SHUHEI KAMEYA, MASATOSHI OHKOSHI, KUNIHIKO YAMAKI,* AND SHOZO SAKURAGI

Department of Ophthalmology, Akita University School of Medicine, Hondo Akita City, Japan 010-8543

Received 14 December 2000/Returned for modification 27 January 2001/Accepted 6 May 2001

We tested 15 adenovirus (Ad)-positive patients involved in a case of nosocomial spread of keratoconjunctivitis. A neutralization test, PCR-restriction fragment length polymorphism analysis, and sequencing of the hypervariable regions of the hexons were performed in order to identify the type of Ad involved. The serotype of the Ad was not identical to any published Ad sequence by either method.

It is well known that some adenovirus (Ad) infections cause severe keratoconjunctivitis, and these infections sometimes cause an outbreak of the disease called epidemic keratoconjunctivitis (EKC). At present, there are 49 identified serotypes of Ad (6, 16, 24), and they have been classified into six subgenera, A to F (21). Ad type 3 (Ad3) and Ad4 are the most common pathogens that cause sporadic keratoconjunctivitis and pharyngoconjunctival fever. Ad8, -19, and -37 have been responsible for sporadic cases as well as outbreaks of severe EKC. They are also known to be the etiological agents for nosocomial infections (7, 10, 22). It is occasionally difficult to make a diagnosis of Ad infection from only the clinical manifestations, and thus, it is important to obtain an accurate and rapid diagnosis of Ad infections by laboratory tests.

The serotype-specific sequences of Ad are distributed as seven discrete hypervariable regions (HVRs) of some hexons in the capsomers (2, 8, 12, 17, 18). It was recently suggested that mutations of a hexon of Ad may play an important role in new outbreaks of Ad infection (17, 19).

In the autumn of 1998, we encountered a case of nosocomial spread of severe keratoconjunctivitis in our hospital. We investigated the serotype of the Ad involved to evaluate whether mutations of the hexon of the Ad contributed to the spread.

All of the subjects were patients in the Akita University School of Medicine hospital, and the keratoconjunctivitis was not limited to a specific population. Samples were collected between September and October 1998 from 15 patients who had typical signs of adenoviral conjunctivitis. The swabbed samples were placed in two different transport media, one for immunochromatography (Adenocheck; Santen, Inc., Osaka, Japan) and the other for cell culture and isolation of the Ad.

The samples were inoculated onto cultures of PHfb cells, MA104 cells, A549 cells, and Hep-2 cells to look for a cytopathic effect. Neutralization tests were performed on A549 cells in 96-well microtiter plates to provide an index of cytopathic effects. The antisera used were those for Ad1, -2, -3, -4, -5, -6, -7, -8, -11, -19, and -37 (SRL Co., Ltd., Tokyo, Japan).

The DNAs of the samples were extracted by standard methods of phenol-chloroform extraction followed by ethanol precipitation. The extracted and purified DNAs were used as templates for PCR-restriction fragment length polymorphism (RFLP) analysis and PCR sequence analysis.

PCR-RFLP analysis was performed according to methods reported previously (1, 3, 4, 9, 14, 15). The amplified and purified DNA was digested with *Eco*T14I, *Hin*fI, and *Hae*III. The patterns of the restriction fragments of the clinical specimens were compared with those of published Ad prototypes (14, 15).

For sequencing of the hexon region of this Ad, 12 primers were synthesized based on the sequence of Ad48 (2) because our virus showed the highest homology with Ad48 when the amplified products of PCR-RFLP analysis were presequenced.

TABLE 1. Primer sets for PCR

Forward		Reverse		Positions
Primer	Sequence	Primer	Sequence	
Ad1F	5′-ATGGCCACCCCCTCGATGCCG-3′	Ad1R	5′-GCAAATGAGCCATAGCATGG-3′	119–758
Ad2F	5′-TCATTTGACATCCGGGGCGTCCTGGA-3′	Ad2R	5′-CTGTCCACCGCAGAGTTCCACAT-3′	418–1237
Ad3F	5′-ATGTGGAACTCTGCGGTGGACAG-3′	Ad3R	5′-GGGATGGGGTAGAGCATGTTGGCGGC-3′	1261–2022
Ad4F	5′-CGATGCTATTGAGAGAGAGG-3′	Ad4R	5′-GTAGGGTTTAAAGCTGGACC-3′	33–421
Ad5F	5′-TCATGTGGTGTACAAGCCAGG-3′	Ad5R	5′-GCGTGGACTTAAAGGAGTCG-3′	997–1551
AdnU-S	5′-TTCCCCATGGCNCACAACAC-3′	AdnU-A	5′-GCCTCGATGACGCCGGCGGTG-3′	1866–2820

* Corresponding author. Mailing address: Department of Ophthalmology, Akita University School of Medicine, Hondo Akita City, Japan 010-8543. Phone: 81-18-884-6167 or -6165. Fax: 81-18-836-2621. E-mail: yamaki@oph.med.akita-u.ac.jp.

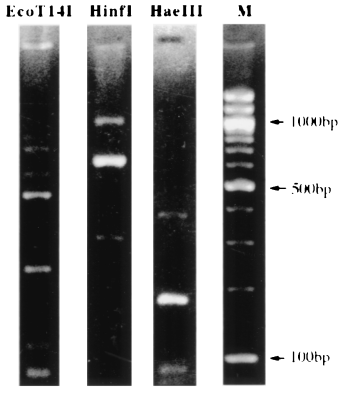

FIG. 1. Agarose gel electrophoresis of PCR-RFLP products showing the cleavage patterns of the 956-bp amplified products. Lane M, molecular standards (100-bp digest).

The primer sets are shown in Table 1. The amplified PCR DNA was subcloned into TOPO TA cloning vector (Invitrogen, Carlsbad, Calif.). The DNA sequencing was done by cycle sequencing according to the method recommended in the instruction manual of the manufacturer (LI COR. Co., Lincoln, Neb.).

Sequence comparison analysis was done using the Internet BLAST search engine (National Center for Biotechnology Information) with the Mac Vector program (Oxford Molecular, Madison, Wis.).

The viruses were isolated from the four cell lines used for the initial isolation. In the neutralization tests, the clinical specimens responded weakly to the antiserum for Ad8 but did not respond to antisera for Ad1, -2, -3, -4, -5, -6, -7, -11, -19, -34, -35, and -37 (Ad9 was not available in Japan).

The PCR products of all clinical specimens showed the same 956-bp length. The restriction patterns of *Eco*T141, *Hin*fI, and *Hae*III showed the same bands for all of the specimens. The restriction pattern of *Eco*T141 showed four bands of approximately 460, 260, 115, and 88 bp. The restriction pattern of *Hin*fI showed two bands of approximately 700 and 250 bp, and that of *Hae*III showed four bands of approximately 400, 180, 95, and 82 bp (Fig. 1).

PCR with the forward primer Ad1F and the reverse primer AdnU-A produced a product of about 2.8 kbp. DNA sequencing revealed a 2,781-bp PCR product (Fig. 2). After the DNA sequences of the seven HVRs were analyzed, a BLAST search was done to find any homology with Ads that have been reported. The sequence homologies of the hexon region are

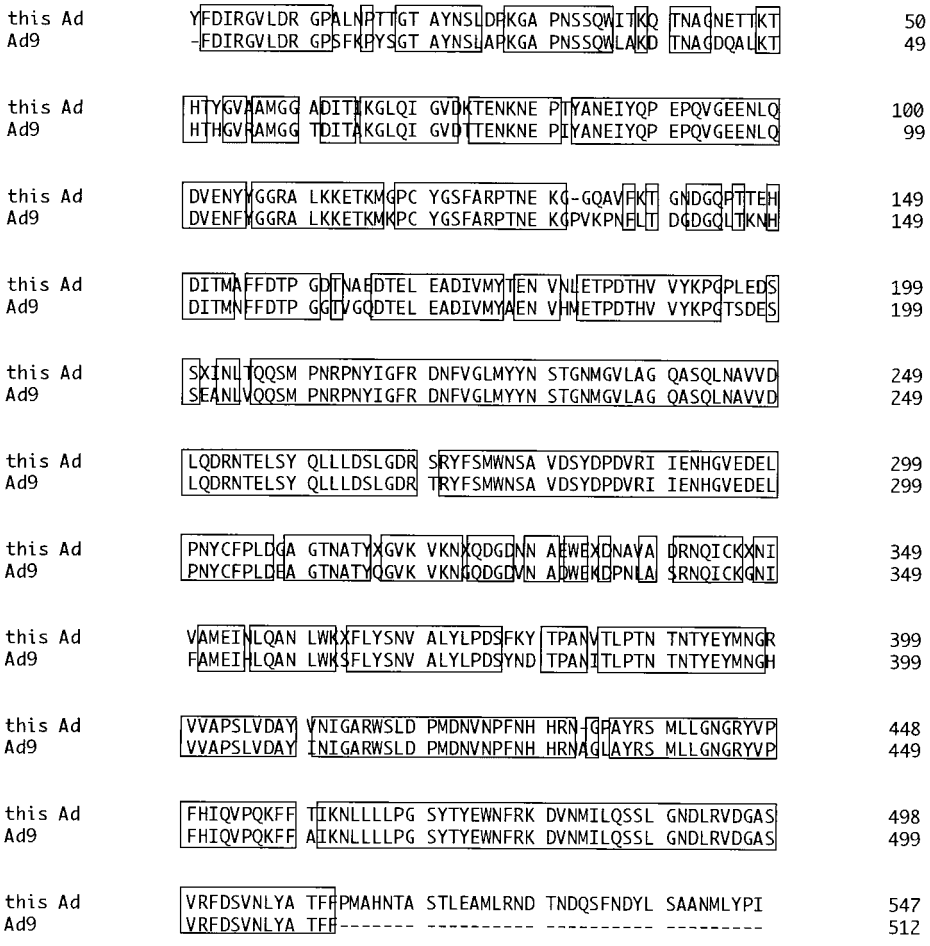

FIG. 2. Comparison of protein sequences of our Ad (this Ad) and Ad9. Identical sequences are boxed.

TABLE 2. Homology between hexon sequences of new Ad and members of Ad subgenus D

Ad	% Homology[a]							
	HVR1	HVR2	HVR3	HVR4	HVR5	HVR6	HVR7	All HVRs
Ad8	15.2	20.0	42.9	50.0	25.0	33.3	41.7	28.9
Ad9	56.3	80.0	85.7	50.0	75.0	40.0	66.7	63.6
Ad15	47.1	40.0	42.9	18.8	52.9	60.0	68.0	49.0
Ad19	40.0	10.0	42.9	43.8	35.0	33.3	58.3	40.6
Ad37	31.6	55.6	40.0	25.0	12.5	55.6	32.0	32.3
Ad48	27.8	16.7	66.7	41.2	21.1	33.3	25.0	29.5

[a] Percent homology between the indicated HVR of the new Ad and that of each subgenus D Ad.

shown in Table 2. An identical Ad type was not found, and the highest homology was with Ad9 (Fig. 2). There was 80% homology with HVR2, 85.7% homology with HVR3, 75% homology with HVR5, and 66.7% homology with HVR7 of Ad9. Lower homologies were found with HVR6 (60.0%) and HVR7 (68.0%) of Ad15 and with HVR3 (66.7%) of Ad48. The homologies of all other HVRs were less than 59%.

In order to identify the type of Ad, we first used a neutralization test, but our Ad was not completely neutralized by the antisera against any prototypes available in Japan. We next used PCR-RFLP (14, 15). The restriction fragment pattern of *Eco*T14I showed a pattern identical to that of Ad4. However, the restriction patterns of *Hin*fI and *Hae*III were not identical to those of any Ad prototypes reported.

We then performed a PCR sequence analysis. The BLAST search of all HVRs showed that an identical sequence had not been reported (Table 2). The highest homology was found with the hexon proteins of Ad9 (Fig. 2). Interestingly, our Ad had the highest homologies primarily with the proteins derived from the D subgenus, Ad9, -15, -37, -8, -19, and -48 (Table 2).

The antigenicity of an Ad is determined by its surface molecules. The sequence encoding the outwardly disposed loops shows considerable variability (11, 20, 21, 23). It was reported that the antigenic specificity of a chimeric Ad was successfully changed by replacing the HVR sequence with those of other serotypes of Ad (5, 13). These results demonstrated that sequence changes in the HVR will change the antigenic specificity of the virus. However, it must be remembered that changing the antigenicities of HVRs creates the possibility of causing an outbreak of infection.

There are two possibile explanations for the outbreak in our hospital. As stated, we found differences in the HVR sequences of this virus and those of Ad9, and it is possible that these differences were caused by an accumulation of mutations. Because mutations of the hexon might change the antigenicity of the Ad, these changes of antigenicity may allow the Ad to escape acquired immunity and may result in an outbreak of EKC.

Another possibility is that our Ad belongs to the D subgenus and its sequence has not been reported. As stated, there are 49 serotypes of Ads (6, 16, 24), and they have been classified into six subgenera (21), A to F. Only 21 sequences of the hexon proteins are reported for 49 Ad serotypes. There are 31 Ad serotypes that belong to the D subgenus, and sequences of hexon proteins from only 8 serotypes have been reported.

In conclusion, it is very important to accumulate information on the relationships among the serotypes, the sequences of their HVRs, and the outbreak of keratoconjunctivitis. These data will be useful in predicting future outbreaks of Ad infection.

REFERENCES

1. **Cooper, R. J., A. C. Yeo, A. S. Bailey, and A. B. Tullo.** 1999. Adenovirus polymerase chain reaction assay for rapid diagnosis of conjunctivitis. Investig. Ophthalmol. Vis. Sci. **40:**90–95.
2. **Crawford-Miksza, L., and D. P. Schnurr.** 1996. Analysis of 15 adenovirus hexon proteins reveals the location and structure of seven hypervariable regions containing serotype-specific residues. J. Virol. **70:**1836–1844.
3. **Dalapathy, S., T. K. Lily, S. Roy, and H. N. Madhavan.** 1998. Development and use of nested polymerase chain reaction (PCR) for the detection of adenovirus from conjunctivitis specimens. J. Clin. Virol. **11:**77–84.
4. **Elnifro, E. M., R. J. Cooper, P. E. Klapper, and A. S. Bailey.** 2000. PCR and restriction endonuclease analysis for rapid identification of human adenovirus subgenera. J. Clin Microbiol. **38:**2055–2061.
5. **Gall, J. G., R. G. Crystal, and E. Falk-Pedersen.** 1998. Construction and characterization of hexon-chimeric adenovirus: specification of adenovirus serotype. J. Virol. **72:**10260–10264.
6. **Hierholzer, J. C., R. Wigand, L. J. Anderson, T. Adrian, and J. W. M. Gold.** 1988. Adenovirus from patients with AIDS: a plethora of serotypes and a description of five new serotypes. J. Infect. Dis. **158:**804–813.
7. **Jernigan, J. A., B. S. Lowry, F. G. Hayden, S. A. Kyger, B. P. Conway, D. H. M. Groschel, and B. M. Farr.** 1993. Adenovirus type 8 epidemic keratoconjunctivitis in an eye clinic: risk factors and control. J. Infect. Dis. **167:**1307–1313.
8. **Kinloch, R., N. Mackay, and V. Mautner.** 1984. Adenovirus hexon: sequence comparison of subgroup C serotype 2 and 5. J. Biol. Chem. **259:**6431–6436.
9. **Li, Q., A. Henningsson, P. Juto, F. Elgh, and G. Wadell.** 1999. Use of restriction fragment analysis and sequencing of a serotype-specific region to type adenovirus isolates. J. Clin. Microbiol. **37:**844–847.
10. **Nauheim, R. C., E. G. Romanowski, T. Araullo-Cruz, R. P. Kowalski, P. W. Turgeon, S. S. Stopak, and Y. J. Gordon.** 1990. Prolonged recoverability of desiccated adenovirus type 19 from various surfaces. Ophthalmology **97:** 1450–1453.
11. **Pring-Åkerblom, P., and T. Adrian.** 1993. The hexon genes of adenoviruses of subgenus C: comparison of the variable regions. Res. Virol. **144:**117–121.
12. **Pring-Åkerblom, P., F. E. J. Trijssenaar, and T. Adrian.** 1995. Sequence characterization and comparison of human adenovirus subgenus B and E hexons. Virology **212:**232–236.
13. **Roy, S., P. S. Shirley, A. McClelland, and M. Kaleko.** 1998. Circumvention of immunity to the adenovirus major coat protein hexon. J. Virol. **72:**6875–6879.
14. **Saitoh, W., N. Itoh, K. Isobe, S. Ohno, A. Oshima, Y. Nagamoto, H. Nakajima, K. Hata, H. Ishiko, and K. Aoki.** 1996. Rapid detection and identification of human adenovirus directly from conjunctival scrapings by polymerase chain reaction and restriction fragment length polymorphism analysis. Nippon Ganka Gakkai Zasshi **100:**163–168.
15. **Saitoh-Inagawa, W., A. Oshima, K. Aoki, N. Itoh, K. Isobe, E. Uchio, S. Ohno, H. Nakajima, K. Hata, and H. Ishiko.** 1996. Rapid diagnosis of adenoviral conjunctivitis by PCR and restriction fragment length polymorphism analysis. J. Clin. Microbiol. **34:**2113–2116.
16. **Schnurr, D. P., and M. E. Dondero.** 1993. Two new candidate adenovirus serotypes. Intervirology **36:**79–83.
17. **Takeuchi, S., A. Oshima, N. Itoh, N. Kitamura, E. Uchio, K. Aoki, and S. Ohno.** 1998. Analysis of adenovirus type 7 hexon hypervariable region. Nippon Ganka Gakkai Zasshi **102:**570–575.
18. **Takeuchi, S., N. Itoh, E. Uchio, K. Aoki, and S. Ohno.** 1999. Serotyping of adenoviruses on conjunctival scrapings by PCR and sequence analysis. J. Clin. Microbiol. **37:**1839–1845.
19. **Takeuchi, S., N. Itoh, E. Uchio, K. Tanaka, N. Kitamura, H. Kanai, K. Isobe, K. Aoki, and S. Ohno.** 1999. Adenovirus strains of subgenus D associated with nosocomial infection as new etiological agents of epidemic keratoconjunctivitis in Japan. J. Clin. Microbiol. **37:**3392–3394.
20. **Toogood, C. I. A., and R. T Hay.** 1988. DNA sequence of the adenovirus type 41 hexon gene and predicted structure of the protein. J. Gen. Virol. **69:**2291–2301.
21. **Toogood, C. I. A., R. Murali, R. M. Burnett, and R. T. Hay.** 1989. The adenovirus type 40 hexon: sequence, predicted structure and relationship to other adenovirus hexons. J. Gen. Virol. **70:**3203–3214.
22. **Warren, D., K. E. Nelson, J. A. Farrar, E. Hurwitz, J. Hierholzer, E. Ford, and L. J. Anderson.** 1989. A large outbreak of epidemic keratoconjunctivitis: problems in controlling nosocomial spread. J. Infect. Dis. **160:**938–943.
23. **Weber, J. M., F. Cai, R. Murali, and R. M. Burnett.** 1994. Sequence and structual analysis of murine adenovirus type 1 hexon. J. Gen. Virol. **75:**141–147.
24. **Wigand, R., T. Adrian, and F. Bricout.** 1987. A new human adenovirus of subgenus D: candidate adenovirus type 42. Arch. Virol. **94:**283–286.