Epidemiol. Infect. (1999), **122**, 281–286. Printed in the United Kingdom

Molecular and serological characterization of adenovirus genome type 7h isolated in Japan

M. HASHIDO[1]*, A. MUKOUYAMA[1], K. SAKAE[2], H. TSUZUKI[2], T. YAMASHITA[2], T. INADA[1] AND S. INOUYE[1]

[1] *Infectious Diseases Surveillance Center, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan*
[2] *Department of Virology, Aichi Prefectural Institute of Public Health, Nagoya, Japan*

(*Accepted 24 October 1998*)

SUMMARY

In 1996, three adenovirus type 7 (Ad7) strains were isolated from children with fever and upper respiratory diseases in Japan. Restriction endonucleases (REs) analysis and PCR amplification of the E3 7·7 kDa ORF revealed that these strains were genotype Ad7h and closely related to an Argentine Ad7h strain, which has been reported to be highly virulent and so far predominant only in South America. These strains showed weak cross-neutralizing activity and specific haemagglutination-inhibition activity to Ad3 antiserum. The present findings suggest that Ad7h in South America has spread to other parts of the world. Since the seroprevalence to Ad7 in the current Japanese population is very low due to the absence of Ad7 circulation in Japan for decades, Ad7 outbreak as a typical case of re-emerging infectious diseases is a cause for serious concern.

INTRODUCTION

Among adenoviruses associated with respiratory infection in humans, adenovirus type 7 (Ad7) is known to be the serotype which most frequently causes severe and even fatal respiratory disease in children. By the use of restriction endonucleases (REs) analysis, this serotype can be further classified as genome types, currently 7p (prototype), 7a, 7b, 7c, 7d, 7e, 7f, 7g, 7h, 7i, and 7j [1–3] according to the nomenclature proposed by Li and Wadell [4]. The genomic analysis of strains collected from around the world and over an extended period of time has revealed that different genomic variants have predominated in different geographic areas and that shifts from one genome type of Ad7 to another have taken place in several areas. Thus the technique has provided a powerful tool for epidemiologic studies. In recent years, there also has been world-wide interest in the use of this technique to investigate the association of the Ad7 genome types with their relative virulence.

At present, the predominant circulating virulent Ad7 genome types associated with respiratory disease in children are Ad7b in Australia, Europe, Brazil, and North America; Ad7c in South Africa; Ad7d in China and Japan, Ad7f in the former Soviet Union, and Ad7h in Argentina, Chile, and Uruguay [2–7]. Of particular interest is the Ad7h, which is currently the predominant virulent genome type in the south cone of South America where it has circulated almost as the sole genomic variant of Ad7 since 1986 after substituting for genome type 7c [3, 8–10]. The Ad7h has been reported to be possibly a highly virulent strain causing severe acute lower respiratory infections in children requiring hospitalization and is frequently detected from fatal cases. Another interesting characteristic of this genome type is that it has been isolated so far only in South American areas.

In the present study, the molecular and serological

* Author for correspondence.

```
                                                                 7.7Kd start
                     10         20         30         40         50 ⇓       60         70
Ad3      1 CAAAAAGGTG ATGCATTACT AAATTTTGAT ATTTAATTTT TTATAGAATT ATGATATTGT TTCAATCAAA
Ad7h     1 CAAAAAGGTG ATGCATTACT AAATTTTGAT GTTTAATTTT TTATAGAATT ATTATATTGT TTCAATCAAA
Ad7p     1 CAAAAAGGTG ATGCATTACT AAATTTTGAC ATTTAATTTT TTATAGAATT ATGATATTGT TTCAATCAAA

Ad3     71 TACCACTA-- ---------- ---------- ---------- ---------- ---------C CTCCTATGCA
Ad7h    71 TACCACTAAC ACTATCAATG TGCAGACTAC TTTAAATCAT GACATGGAAA ACCACACTAC CTCCTATGCA
Ad7p    71 TACCACTAAC ACTATCAATG TGCAGACTAC TTTAAATCAT GACATGGAAA ACCACACTAC CTCCTATGCA

Ad3     90 TACACAAACA TTCAGCCTAA ATACGCTATG CAACTAGAAA TCACAATACT AATTGTAATT GGAATTCTTA
Ad7h   141 TAC------- ---------- ---------- ---------- ---------- ---------- ----------
Ad7p   141 TACATAAACA TTCAGCCTAA ATACGCTATG CATCTAAAAA TCACCATACT AATTGTAATT GGAATTCTTA

Ad3    160 TACTATCTGT TATTCTTTAT TTTATATTCT GCCGTCAAAT ACCCAATGTT CATAGAAATT CTAAAAGACC
Ad7h       ---------- ---------- ---------- ---------- --CCAATGTT CATAGAAATT CTAAAAGACG
Ad7p   211 TACTATCTGT TATTCTTTAT TTTCTATTCT ---------- ---------- ---------- ----------

Ad3    230 TCCCATCTAT TCTCCTATGA TTAGTCGTCC CCATATGGCT CTGAATGAAA TCTAAGATCT TTTTTTTT-C
Ad7h   172 TCCCATCTAT TCTTCTATGA TTAGTCGTCC CCATATGGCT CTGAATGAAA TCTAAGATCT TTTTTTTT-C
Ad7p       ---------- ---CCTATGA TTAGTCGTCC CCATATAGCT CTGAATGAAA TCTAAGATCT TTTTTTTTTC

Ad3    299 TCTTACAGTA TGGTGAACA  317
Ad7h   241 TCTTACAGTA TGGTGAACA  259
Ad7p   298 TCTTACAGTA TGGTGAACA  316
```

Fig. 1. DNA sequence comparison of the 7·7 kDa ORF in the E3 region among Ad3, Ad7h, and Ad7p. Squares indicate the sites for setting the pair of primers.

characteristics of the Ad7h strains, which were isolated in Japan from children with fever and upper respiratory disease [11], were investigated.

MATERIALS AND METHODS

Viruses

The three Ad7 strains, designated as 96A90, 96A799 and 96A2937, were isolated in 1996 from children of 1, 5 and 7 years of age, who presented with rash, fever, and upper respiratory diseases, in Aichi prefecture located at the mid-west part of Japan [11]. For the comparison, Ad7p (Gomen), Ad7a (S1058) and Ad3p (G.B.) strains obtained from ATCC (Rockville, MD), and Japanese Ad7 strains 383 and Bal (both Ad7d with an only different RE pattern by *Bst*EII), which were isolated in 1992 and 1995, respectively, were also included in the study. The clinical isolates were propagated in HEp-2 or A-549 cell lines and strain-purified by plaque formation.

DNA restriction analysis

Intracellular viral DNA was extracted from infected cells with a modified Hirt procedure [12]. Sixteen of six bp-recognizing endonucleases, *Bam*HI, *Bcl*I, *Bgl*I, *Bgl*II, *Bst*EII, *Dra*I, *Eco*RI, *Hpa*I, *Hin*dIII, *Nru*I, *Pst*I, *Sal*I, *Sma*I, *Ssp*I, *Xba*I and *Xho*I were purchased from Toyobo (Tokyo, Japan) and two REs, *Asn*I and *Bfr*I from Boehringer (Mannheim, FRG). All enzyme reactions were carried out according to the manufacturers' instructions. The DNA fragments were separated by electrophoresis in 1% SeaKem GTG agarose (FMC BioProducts, Rockland, ME) horizontal gels which were prepared and run in 40 mM Tris-acetate buffer at 50 V. Gels were stained in ethidium bromide (1 μg/ml). Photography was performed with a Fluorescein Analyzer System (Toyobo).

PCR amplification

According to the sequences published elsewhere [13, 14], the genome size of the 7·7 kDa ORF in the E3 region of Ad3, 7p and 7h are variable due to deletions of different width (Fig. 1). Therefore, a primer pair using two short sequences neighbouring the 7·7 kDa ORF which are common among Ad3, 7p and 7h was prepared and used to estimate the genome size of the 7·7 kDa ORF of clinical isolates 90, 799 and 2937.

Forward primer:
5′-CAAAAAGGTGATGCATTAC-C′-3′
Reverse primer:
5′-TGTTCACCATACTGTAAGA-3′

The 35 PCR cycles were performed according to a standard method at 94 °C for 30 s, 50 °C for 90 s, and 72 °C for 2 min using SuperTaq Premix Kit (Sawady

Technology, Tokyo, Japan) and a Perkin–Elmer Cetus Thermal Cycler 480 (Emeryville, CA). The products were separated by electrophoresis in 3% NuSieve agarose (FMC BioProducts) gels, and then stained and photographed as described above. The PCR marker (Novagen, Madison, WI) was used as a size standard for determination of the molecular weight of the products.

Serological tests

The neutralization test (NT) was performed in microtitre plates using A-549 cells by conventional means. Briefly, adequately diluted virus and twofold dilutions of serum, both in 25 μl volumes, were incubated in microplates at 37 °C for 90 min. Then 0·1 ml of cell suspension were added and the incubation was continued until the results were read in 3 days. The haemagglutination-inhibition (HI) test was also carried out in microtitre plates with the use of blood cells of rhesus monkey. Briefly, 4 HA unit of virus and twofold dilutions of serum which was treated to remove non-specific agglutinins beforehand, both in 25 μl volumes, were incubated at room temperature for 1 h. Then 50 μl of 0·4% erythrocytes was added and the mixture was allowed to stand for 1·5 h at 37 °C, after which the results were read. The rabbit antisera to Ad3 and Ad7 used in the both tests were obtained from ATCC. The rhesus blood cells were kindly supplied by Drs Ryozaburo Mukai and Akio Yamada, Tsukuba Primates Center, NIID, Tsukuba, Japan.

RESULTS

Identification of the genome type of clinical isolates 90, 799 and 2937

The DNA restriction profiles after cleavage with *Bam*HI are shown in Figure 2. The profiles of 90, 799 and 2937 were different from those of Ad3p, 7p, 7a, and recently circulating Japanese strains, 7d (383 and Bal), but were found to correspond to the pattern reported for the genome type Ad7h which was first isolated in Argentina in 1984 and has been isolated so far only in South America. According to the classification proposed by Li and Wadell [4], the strains 90, 799 and 2937 were determined as genome type Ad7h.

The DNA restriction patterns of 90, 799 and 2937 were the same as Ad7h with all enzymes used here, except for *Hin*dIII (Fig. 3); with *Hin*dIII, one restriction site was lost in 2937, which resulted in the loss of the 2·2K and 3·3K DNA fragments and the gain of a 5·5K fragment.

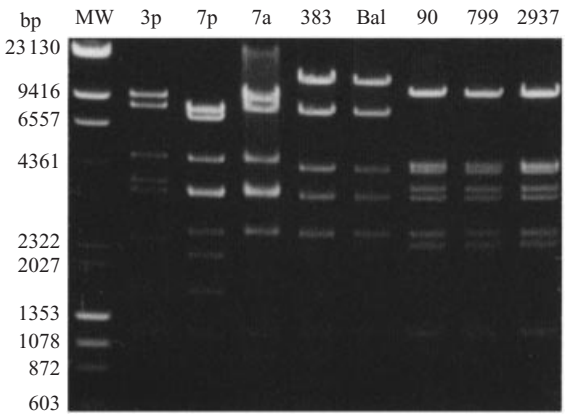

Fig. 2. Comparison of the *Bam*HI restriction patterns among Ad3 and Ad7 genome types.

Among the 18 RE profiles for strains 90, 799 and 2937, 13 were identical to those for Ad7d (383) (Fig. 3), although 5 profiles were different from them, i.e. *Bcl*I, *Bgl*II, *Pst*I, *Xho*I (Fig. 4), and *Bam*HI (Fig. 2). In contrast, comparison of the 18 RE profiles with the profiles of Ad7h in South America given in the literature [8, 15], showed there existed only one different cleavage pattern (with *Xho*I).

Comparison of the genome size of the E3 7·7 kDa ORF among Ad3, 7p and clinical isolates 90, 799 and 2937

The size of PCR products of the 7·7 kDa ORF in the E3 region of 90, 799 and 2937 were compared to those of Ad3 and 7p (Fig. 5). The molecular weight of the products for 90, 799 and 2937, which showed the same size, was estimated to be about 260 bp, smaller than those of Ad3 (317bp) and 7p (316bp) but close to that of Ad7h in South America (259bp) as calculated from the sequences shown in Figure 1.

NT and HI analysis

Table 1 shows the results of NT and HI titrations for Ad3 and various Ad7 strains against Ad3p and Ad7a antisera. The strains Ad7p, 7a, 383 and Bal reacted to Ad7 antiserum and Ad3p reacted to Ad3 antiserum specifically, both in NT and HI. In contrast, the strains 90, 799 and 2937 reacted mainly to Ad7

https://doi.org/10.1017/S0950268899002149 Published online by Cambridge University Press

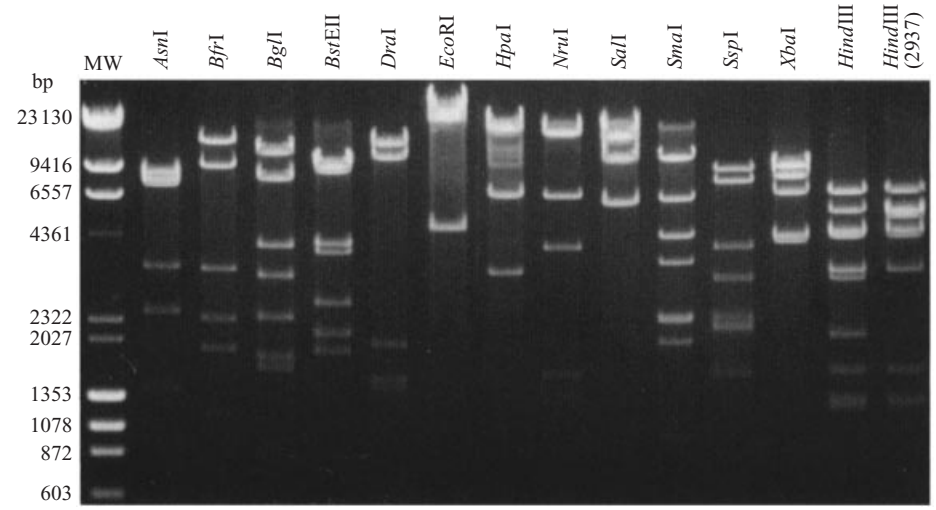

Fig. 3. DNA restriction profiles of 96A90 obtained after cleavage with *Asn*I, *Bfr*I, *Bgl*I, *Bst*EII, *Dra*I, *Eco*RI, *Hpa*I, *Nru*I, *Sal*I, *Sma*I, *Ssp*I, *Xba*I, and *Hin*dIII. They were all identical with those of strains Ad7d (383), 96A799, and 96A2937, except for *Hin*dIII (the last lane).

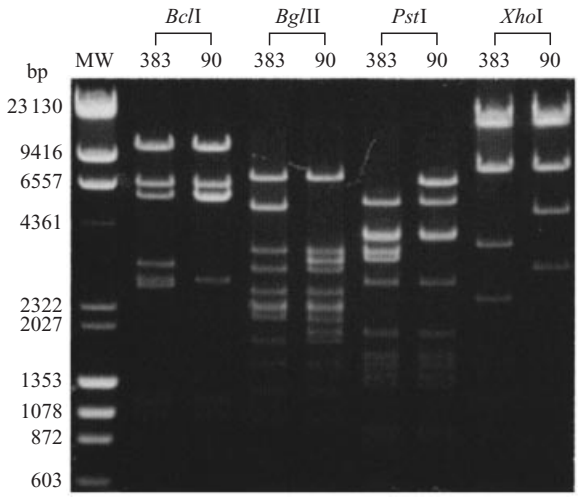

Fig. 4. DNA restriction profiles of Ad7d (383) and 96A90 obtained after cleavage with *Bcl*I, *Bgl*II, *Pst*I, and *Xho*I.

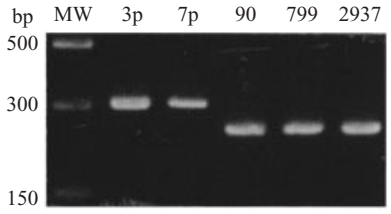

Fig. 5. Comparison of the size of PCR products of the 7·7 kDa ORF in the E3 region among Ad3, 7p, and Japanese strains 96A90, 96A799 and 96A2937.

antiserum in NT, but in HI, they showed specific reactivity only to Ad3 antiserum. In NT, they also showed cross-neutralizing activity to Ad3 antiserum, to a small extent though.

DISCUSSION

The RE analysis showed that the Ad7h was isolated in Japan in 1996 from children with fever and upper respiratory disease. Furthermore, these viruses have genomic and serologic characteristics similar to those of the Ad7h strain circulating in the south cone of South America, suggesting the possibility that the origin of the Japanese Ad7h strains might be South America.

First, 17 REs out of 18, except for *Xho*I, showed the common restriction profiles between the Japanese clinical isolates 90, 799 and 2937 (named tentatively here as Aichi strains) and the Ad7h strain in South America (abbreviated tentatively as Ad7h/Arg), thus indicating that the Aichi strains are closely related to the Ad7h/Arg. Although the predominant virulent Ad7 genome type in Japan has been Ad7d since the late 1980s [7], five RE patterns of the Aichi strains were different from those of Ad7d, which seems to suggest that it is unlikely that the Aichi strains have evolved from the pre-circulating Ad7d strains through mutations.

Secondly, the size of PCR products of the 7·7 kDa ORF of Aichi strains estimated from gels was smaller than that of Ad7p and about the same as that of Ad7h/Arg. Considering that the deletion in the 7·7 kDa ORF of the E3 region leading to a size of 259 bp is one of the unique characteristics of Ad7h/Arg [14], this finding also indicates that the Aichi strains and Ad7h/Arg are genetically very close.

Thirdly, the Aichi strains showed HI activity specific to Ad3, and neutralizing activity to both Ad7

https://doi.org/10.1017/S0950268899002149 Published online by Cambridge University Press

Table 1. *NT and HI titres of Ad3 and Ad7 strains*

Adenovirus	Ad3p	Ad7p	Ad7a	383	Bal	90	799	2937
NT titration								
Ad3p antiserum	320	< 5	< 5	< 5	< 5	20	20	20
Ad7a antiserum	< 5	320	640	640	1280	1280	1280	640
HI titration								
Ad3p antiserum	320	< 5	< 5	< 5	< 5	160	320	160
Ad7a antiserum	< 5	320	1280	640	320	< 5	< 5	< 5

and Ad3. These findings also support our hypothesis, because (1) Kajon and colleagues reported that the sequence of the fibre gene of Ad7h/Arg is more homologous to Ad3 than Ad7p (98·2 *vs.* 66·6%) [14], and (2) although no data on cross-neutralizing activity to Ad3 has yet been published for Ad7h/Arg, the fact that this strain has been first wrongly serotyped as Ad3 [8, 15] leads us to surmize that Ad7h/Arg showed a certain level of reactivity to the Ad3 antiserum. The fibre of adenovirus is known to have the haemagglutinating domain and also contain epitopes recognized by neutralizing antibodies [16]. If the fibre gene of the Aichi strains had been replaced by that of Ad3, as happened in the Ad7h/Arg, then the HI activity and cross-neutralizing activity of the Aichi strains to Ad3 may be reasonably explained. However, one observation by us on the Aichi strains which was different from those on Ad7h/Arg, was that Aichi strains clearly showed intact HA activity. Kajon and Wadell suggested that, in Ad7h/Arg, a mismatch from the Ad3 sequence in the haemagglutinating domain in the knob region of the fibre had resulted in the loss of HA activity [14]. To investigate this difference, sequence analysis of the fibre gene of the Aichi strains is under progress in our laboratory.

According to the National Epidemiological Surveillance of Infectious Diseases (NESID) in Japan, the marked epidemiological characteristics of the adenovirus subgenus B in Japan for decades is that Ad3 was the most prevalent type and Ad7, which is often detected in other countries, was isolated very rarely [17]. During the 14 years from 1980 to 1993, reports on isolation of Ad3 numbered 7187, in contrast to only 30 reports of Ad7 [18]. The long absence of Ad7 circulation in Japan should have led to a decrease in the antibody prevalence and emerging epidemics. In 1994, the seroprevalence to Ad7 was < 5% in the general population < 40 years of age [19–21]. From April 1995, Ad7 began to be isolated successively all over Japan and number of reports to NESID had reached 274 by December 1996 [22]. The RE analysis revealed that all of the newly emerging epidemic Ad7 strains are a new variant of genome type Ad7d [7], which has predominated in China since 1980 [4], and has also been found in Japan since the late 1980s.

Thus, not only for Ad7d, but also for Ad7h, the current serostatus to Ad7 among Japanese may provide a hotbed of emerging epidemics. Although the cases presented here were not fatal, we have become anxious recently because isolation of Ad7 strains which showed cross-reactivity to Ad3 has been reported more frequently and some of them were involved in fatal or severe cases with lower respiratory diseases (investigation in progress).

The present study would give warning to the international community by (1) showing that Ad7h, a virulent strain which has been confined only to South America so far, is now giving signs of spreading out to other parts of the world, and (2) discussing the Ad7 outbreak crisis in Japan as a typical model of re-emerging infectious diseases. Continuing surveillance along with genome type analysis and the study of its clinical significance are necessary to control its epidemics and to elucidate the pathogenicity of Ad7 infection.

ACKNOWLEDGEMENTS

We would like to thank Drs Ryozaburo Mukai and Akio Yamada, Tsukuba Primate Center, NIID, for kindly supplying blood cells of rhesus monkeys. This work was supported in part by the Special Research Project of NIID.

REFERENCES

1. Li Q, Wadell G. Analysis of 15 different genome types of adenovirus type 7 isolated on five continents. J Virol 1986; **60**: 331–5.
2. Golovina G, Zolotaryov F, Yurlova T. Sensitive

analysis of genetic heterogeneity of adenovirus types 3 and 7 in the Soviet Union. J Clin Microbiol 1991; **29**: 2313–21.
3. Kajon A, Mistchenko A, Videla C, Hortal M, Wadell G, Avendano L. Molecular epidemiology of adenovirus acute lower respiratory infections of children in the south cone of South America (1991–1994). J Med Virol 1996; **48**: 151–6.
4. Li Q, Zheng Q, Liu Y, Wadell G. Molecular epidemiology of adenovirus types 3 and 7 isolated from children with pneumonia in Beijing. J Med Virol 1996; **49**: 170–7.
5. Wadell G. Molecular epidemiology of human adenoviruses. Curr Top Microbiol Immunol 1984; **110**: 191–220.
6. Adrian T, Becker M, Hierholzer J, Wigand R. Molecular epidemiology and restriction site mapping of adenovirus 7 genome types. Arch Virol 1989; **106**: 73–84.
7. Noda M, Kiritani M, Abe K, Ikeda Y, Yamaoka K, Ogino T. Restriction enzyme analysis of adenovirus type 7 isolates in Japan. IASR, National Institute of Health and Ministry of Health and Welfare, Japan 1996; **17**: 102–4.
8. Kajon A, Murtagh P, Garcia Franco S, Freire M, Weissenbacher M, Zorzopulos J. A new genome type of adenovirus 3 associated with severe lower acute respiratory infection in children. J Med Virol 1990; **30**: 73–6.
9. Kajon A, Wadell G. Genome analysis of South American adenovirus strains of serotype 7 collected over a 7-year period. J Clin Microbiol 1994; **32**: 2321–3.
10. Niel C, Moraes M, Mistchenko A, Leite J, Gomes S. Restriction site mapping of four genome types of adenovirus types 3 and 7 isolated in South America. J Med Virol 1991; **33**: 123–7.
11. Sakae K. Isolation of adenovirus type 7 in 1996 – Aichi. IASR, National Institute of Infectious Diseases and Ministry of Health and Welfare, Japan 1997; **18**: 82–6.
12. Wadell G, de Jong J. Restriction endonucleases in identification of a genome type of adenovirus 19 associated with keratoconjunctivitis. Infect Immun 1980; **27**: 292–6.
13. Signas C, Akusjarvi G, Pettersson U. Region E3 of human adenoviruses; differences between the oncogenic adenovirus-3 and the non-oncogenic adenovirus-2. Gene 1986; **50**: 173–84.
14. Kajon A, Wadell G. Sequence analysis of the E3 region and fiber gene of human adenovirus genome type 7h. Virol 1996; **215**: 190–6.
15. Kajon A, Wadell G. Characterization of adenovirus genome type 7h: analysis of its relationship to other members of serotype 7. Intervirol 1992; **33**: 86–90.
16. Horwitz M. Adenoviruses. In: Fields B, Knipe D, Howley P, eds. Fields virology. Philadelphia: Lippincott–Raven, 1996: 2149–71.
17. Yamadera S, Yamashita K, Akatsuka M, et al. Adenovirus surveillance, 1982–1993, Japan. A report of the National Epidemiological Surveillance of Infectious Agents in Japan. Jpn J Med Sci Biol 1995; **48**: 199–210.
18. Infectious Diseases Surveillance Center, National Institute of Infectious Diseases and Ministry of Health and Welfare, Japan. Emergence of adenovirus type 7, Japan, 1995. IASR 1996; **17**: 99–100.
19. Ozawa S, Yamagami T, Watanabe Y, Machida A, Yakutai K, Yokoyama H. Serosurvey after an outbreak of adenovirus type 7 infection at a high school dormitory – Yamanashi. IASR, National Institute of Infectious Diseases and Ministry of Health and Welfare, Japan 1997; **18**: 82–3.
20. Kajiwara J, Hamasaki M, Ishibashi T, Chijiwa K, Fukuyoshi S, Mori R. Analysis of genome types of adenovirus type 7 isolated in Fukuoka Prefecture in 1996. J Jap A Infect Dis 1997; **71**: 895–8.
21. Miwa C. Serosurvey for adenovirus type 3 and adenovirus type 7 infection in resident in Gifu prefecture, 1996. J Jap A Infect Dis 1997; **71**: 1261–2.
22. Infectious Diseases Surveillance Center, National Institute of Infectious Diseases and Ministry of Health and Welfare, Japan. Adenovirus type 7, Japan, April 1995–December 1996. IASR 1997; **18**: 79–80.