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Review

Human adenovirus type 8 genome typing

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Human adenovirus type 8 (HAdV-8) is a major causative agent of epidemic keratoconjunctivitis, which is frequently associated with community, industrial and nosocomial outbreaks. Restriction endonuclease (RE) analysis discriminates HAdV-8 isolates into genome types, making it possible to correlate between genomic variants, virulence and outbreak potential. RE analysis is performed using two sets of classification criteria, an Asian and a European system. So far, genome types HAdV-8A–8K and HAdV-8/D1–D12 have been included in the Asian and European classifications, respectively. Conventionally followed RE analysis has some inherent problems, such as the use of a neutralization test for HAdV-8 typing, which may misidentify some recombinant adenoviruses as HAdV-8 due to cross-reaction, the lack of a complete restriction profile for all genome types for purposes of comparison, and the absence of enzyme codes in the Asian classification system. In this review, we propose typing of HAdV-8 with phylogenetic analysis of the hexon and fibre genes prior to RE analysis due to the emergence of many recombinant types. Schematic restriction profiles for both classification systems were created by compiling all the published reports on genome types, and enzyme codes were included for the Asian classification system. The updated and simplified stepwise approach for HAdV-8 genome typing presented here could be useful for identifying either existing genome types or novel ones.

Introduction

Human adenovirus type 8 (HAdV-8) is frequently associated with epidemic keratoconjunctivitis (EKC), a severe and highly contagious eye disease (Shenk, 1996; Paval-Langston, 1994). Being a non-enveloped virus, HAdV-8 is remarkably resistant to physical and chemical agents and remains infectious at room temperature for prolonged periods, thereby facilitating transmission through fomites, ophthalmic instruments and even ocular drops (Hamada *et al.*, 2008; Saitoh-Inagawa *et al.*, 1999). This endows the virus with the potential to spread in the community as well as in medical facilities.

Conventional typing with a neutralization test (NT) cannot differentiate genetic diversity among the field strains of HAdV-8 (Hierholzer *et al.*, 1991; Wadell *et al.*, 1980). Genome typing by restriction endonuclease (RE) cleavage pattern analysis of isolates is the method of choice for elucidating this diversity. This method enables prediction of a possible correlation between genomic variation and virulence of the strain. So far, HAdV-8A–8K and HAdV-8/D1–D12 have been described using the Asian and European classification systems of genome typing, respectively (Fujii *et al.*, 1983, 1984; Fujii & Yamashita, 1984; Ishii *et al.*, 1987; Sawada *et al.*, 1987; Sheu *et al.*, 1987; Guo *et al.*, 1988; Chang *et al.*, 2001; Adhikary *et al.*, 2003, 2011; Ohguchi *et al.*, 2003; Jin *et al.*, 2011; Takács *et al.*, 1983; Adrian *et al.*, 1990; de Jong *et al.*, 1992; Tanaka *et al.*, 2000). Nevertheless, there have also been several problems with genome typing, such as the application of variable numbers and types of REs as well as difficulties in the interpretation of results due to the lack of a published catalogue of HAdV-8 genome types. The aim of this review is to develop an updated organized approach to HAdV-8 genome typing by compiling all the published information. The resulting data are expected to provide support for future epidemiological studies.

Historical overview

During the summer of 1941, a very large outbreak (more than 10 000 cases) of keratoconjunctivitis occurred in Hawaii, at the naval shipyards of Pearl Harbor. Soon thereafter, it spread to the West Coast of the United States and seriously hampered wartime industrial production.

Table 1. Human adenovirus 8 genome type circulation: classification introduced by Fujii *et al.* (1983)

Reference	Year and country of isolation	No. of isolates	Restriction enzyme*							Period and place of circulation	Genome type
			*Bam*HI	*Hin*dIII	*Kpn*I	*Pst*I	*Sac*I	*Sal*I	*Sma*I		
Fujii *et al.* (1983)	1975–1981, Japan	25	√	√	x	√	x	√	x	1975–1978, Sapporo	HAdV-8A
										1976–1981, Sapporo	HAdV-8B
Fujii *et al.* (1984)	1980–1981, Taiwan	27	√	√	x	√	√	√	x	1980–1981, Kaohsiung	HAdV-8C, D, E, F
Kemp & Hierholzer (1986)	1966–1985, United States, Taiwan, Greece	3	x	√	√	x	√	√	√	1966–1970, Georgia, Illinois	HAdV-8P
		21	x	√	√	x	√	√	√	1971–1972, Ohio, Alabama, Missouri, Maryland	HAdV-8C
		4	x	√	√	x	√	√	√	1972, Maryland	HAdV-8P
		6	x	√	√	x	√	√	√	1973–1974, Tennessee	HAdV-8C
		15	x	√	√	x	√	√	√	1975, Vietnam, Florida	HAdV-8D
		13	x	√	√	x	√	√	√	1977–1985, Georgia, Virginia, Massachusetts, Florida, Tennessee, Louisiana	HAdV-8P
		3	x	√	√	x	√	√	√	1980–1983, Athens	HAdV-8P
		22	x	√	√	x	√	√	√	1981–1983, Taipei	HAdV-8P
Sawada *et al.* (1987)	1977–1981, Japan, Philippines	5	x	√	x	x	x	√	√	1983–1984, Sapporo	HAdV-8B
		3	x	√	x	x	x	√	√	1983–1984, Manila	HAdV-8P
Ishii *et al.* (1987)	1980–1983, Japan, Taiwan South Korea	7	√	√	x	x	√	√	x	1983, Sapporo	HAdV-8B
		6	√	√	x	x	√	√	x	1983, Kaohsiung	HAdV-8C, E
		9	√	√	x	x	√	√	x	1983, Bussan	HAdV-8E
Sheu *et al.* (1987)	1983–1984, Taiwan	18	√	√	x	√	√	√	√	1983–1984, Kaohsiung	HAdV-8C, E, G
Guo *et al.* (1988)	1973–1986, Japan Australia Philippines	11	x	√	x	x	x	√	x	1984–1986, Sapporo	HAdV-8B
		5	√	√	x	x	x	√	x	1984–1986, Australia	HAdV-8P
		4	√	√	x	x	x	√	x	1984, Philippines	HAdV-8P
McMinn *et al.* (1991)	1989, Australia	18	x	√	√	x	√	√	√	1989, Alice Springs	HAdV-8P, C?
Chang *et al.* (2001)	1990–1994, Taiwan	21	√	√	x	√	√	√	√	1990–1994, Kaohsiung	HAdV-8H
Ohguchi *et al.* (2003)	2002, Bangladesh	9	√	√	x	√	√	√	√	2002, Chittagong	HAdV-8P, E, 8?
Adhikary *et al.* (2003)	1983–1997, Japan	13	√	√	x	√	√	√	√	1983–1988, Hiroshima	HAdV-8A
		7	√	√	x	√	√	√	√	1983–1988, Hiroshima	HAdV-8B
		35	√	√	x	√	√	√	√	1984–1995, Hiroshima	HAdV-8E
		74	√	√	x	√	√	√	√	1995–1997, Hiroshima	HAdV-8I

Table 1. cont.

Reference	Year and country of isolation	No. of isolates	Restriction enzyme*						Period and place of circulation	Genome type
			*Bam*HI	*Hin*dIII	*Kpn*I	*Pst*I	*Sac*I	*Sal*I	*Sma*I	
Adhikary *et al.* (2011)	1998–1999, Japan	26	√	√	x	√	√	√	√	1998, Miyaknonojyo 1999, Miyaknonojyo	HAdV-8E, J HAdV-8E
Jin *et al.* (2011)	1986–1996, Japan	1	√	√	x	√	√	√	√	1986, Sapporo	HAdV-8A
		2	√	√	x	√	√	√	√	1986, Sapporo	HAdV-8B
		3	√	√	x	√	√	√	√	1991, Sapporo	HAdV-8K
		3	√	√	x	√	√	√	√	1996, Sapporo	HAdV-8E

*√, Enzyme used in the study; x, enzyme not used in the study.

Several thousand cases were diagnosed and most of them were in shipyard workers. The disease was thus named 'shipyard eye' (Jawetz, 1959). Subsequently, due to its epidemic nature, the illness was called 'epidemic kerato-conjunctivitis' (EKC) (Hogan & Crawford, 1942). The disease was characterized by severe bilateral conjunctivitis with a painful and distressing keratitis, severe photophobia and corneal opacities with variable degree of visual impairment for months to years (Jawetz *et al.*, 1955a, 1955b, 1957). EKC was also described among German workers in the late 19th century. However, the aetiological agent remained unidentified until December 1954, when a seaman travelling from an East Asian port came to an eye clinic of the University of California for treatment of severe bilateral conjunctivitis (red eye). A conjunctival scraping was collected from the seaman (whose surname was Trim) and the causative virus of 'shipyard eye' was isolated for the first time in HeLa cell culture. The virus was typed as HAdV-8 by homologous antiserum and designated the prototype strain Trim (Jawetz *et al.*, 1955a, b, 1957). Since its original isolation, HAdV-8 remains the major agent of EKC around the world and has been isolated from many outbreaks in community, industrial and medical settings, including the offices of ophthalmologists (Guyer *et al.*, 1975; Laibson *et al.*, 1968; Sprague *et al.*, 1973; Dawson & Darrell, 1963; Patrick & Matthews, 1981; Buehler *et al.*, 1984; D'Angelo *et al.*, 1981). However, the intraserotypic genetic variability of HAdV-8 field isolates was not examined until 1983, when the viral DNA was digested by multiple REs and genetically different strains were described as genome types. Two genome type classification systems were developed separately in Asia and Europe.

Genome type designation based on alphabetical order: Asian classification

Fujii *et al.* (1983) used a nomenclature system of HAdV-8 genome types that were denominated alphabetically. The letter P was assigned to the prototype strain Trim, and the letters A, B, C, D, E, etc., were assigned to the rest. So far, strains HAdV-8A–HAdV-8K have been identified as the result of a series of studies among Asian countries (Table 1) (Fujii et *al.*, 1983, 1984; Sheu *et al.*, 1987; Chang *et al.*, 2001; Adhikary *et al.*, 2011; Jin *et al.*, 2011). The genome types do not always correlate with the chronological isolation of the strain. For example, HAdV-8E was originally isolated in 1980 before HAdV-8D in 1981 and HAdV-8K was isolated in 1991 before HAdV-8J in 1998. In this classification system, *Sac*I is the most discriminating enzyme followed by *Hin*dIII and *Sal*I.

Fujii *et al.* (1983) for the first time applied RE analyses using *Bam*HI, *Hin*dIII, *Pst*I and *Sal*I to 25 ocular isolates and observed cleavage patterns different from those for the HAdV-8 prototype (strain Trim, HAdV-8P). They designated these isolates the genome types HAdV-8A and HAdV-8B. HAdV-8A was detectable from 1975 through 1978, and HAdV-8B was detectable from 1976 through

1981 in the population of Sapporo, Japan. Subsequently, in a collaborative study between Japan and Taiwan, 27 strains isolated between July 1980 and July 1981 in Kaoshiung, Taiwan, were analysed with *Bam*HI, *Hin*dIII, *Pst*I, *Sac*I and *Sal*I and four new genome types, HAdV-8C–8F, were discovered (Fujii *et al.*, 1983, 1984). In 1987, a novel genome type, HAdV-8G, was discovered by analysis with *Bam*HI, *Hin*dIII, *Pst*I, *Sac*I, *Sal*I and *Sma*I that was co-circulating with HAdV-8C and HAdV-8E in Kaohsiung from 1983 to 1984 (Sheu *et al.*, 1987). Afterwards, 21 strains isolated in Kaohsiung from January 1990 to December 1994 were cleaved with *Bam*HI, *Hin*dIII, *Pst*I, *Sal*I, *Sma*I and *Sst*I, and a new genome type, HAdV-8H, was discovered (Chang *et al.*, 2001). One hundred and twenty-nine strains isolated from an outbreak as well as sporadic cases of EKC in Hiroshima City, Japan, over a 15-year period (1983–1997) revealed a novel genome type, HAdV-8I, and the previously described genome types HAdV-8A, HAdV-8B and HAdV-8E. The new genome type HAdV-8I was isolated from an outbreak of EKC in 1995 and thereafter from sporadic cases until 1997 (Adhikary *et al.*, 2003). In the years 1998–1999, 26 isolates from Miyakonojyo, Japan, were analysed with *Bam*HI, *Hin*dIII, *Pst*I, *Sac*I, *Sal*I and *Sma*I and identified as HAdV-8E along with a single strain of novel genome type HAdV-8J that was recognized from a sporadic case of EKC (Adhikary *et al.*, 2011). Chronological changes in HAdV-8 genome type circulation were observed from 1986 to 2003 in Sapporo, Japan, and a new genome type, HAdV-8K, was isolated in 1991 (Jin *et al.*, 2011).

Genome type designation based on numerical order: European classification

A numerical code to denominate HAdV-8 genome types was proposed by Adrian *et al.* (1990). The formula for designating a genome type included five pieces of information as follows: number of serotype/number of variant/designation of first identified strain of variant/geographical origin of this strain/year of isolation of this strain. Adrian *et al.* (1990) used six RE patterns in alphabetical order (*Bam*HI, *Bgl*II, *Hin*dIII, *Kpn*I, *Sal*I and *Sma*I). Later, *Bgl*I, *Bst*EII and *Sac*I were included in successive studies (de Jong *et al.*, 1992; Tanaka *et al.*, 2000). The cleavage pattern of the prototypes for all REs was labelled as enzyme code 1. Patterns deviating from those of the prototype were named 2, 3, etc., depending on the chronological order of the respective isolates. For example, the DNA variant HAdV-8 described by Adrian *et al.* (1990) was referred to as HAdV-8/D1/Koja/Japan/61 and was abbreviated as HAdV-8/D1. HAdV-8/D1 is, by definition, the DNA variant to which prototype strain Trim belongs. So far, HAdV-8/D1–HAdV-8/D12 have been described (Table 2) (Adrian *et al.*, 1990; de Jong *et al.*, 1992; Tanaka *et al.*, 2000). In this classification system, *Bgl*I and *Hin*dIII are the most discriminating enzymes, followed by *Bam*HI, *Bgl*II and *Sal*I.

Early HAdV-8 genome type classification was begun in Europe using the numerical codes of restriction enzymes to denominate the adenovirus genome type in chronological order of the isolates (Adrian *et al.*, 1990). Six REs (*Bam*HI, *Bgl*II, *Hin*dIII, *Kpn*I, *Sal*I and *Sma*I) were applied to 60 strains of HAdV-8 that were isolated from Germany, Japan, Austria and Hungary over a 20-year period from 1961 to 1982. Fifty-four strains were isolated in Germany and 6 from other countries. Forty-five of these isolates were from the same source and classified as genome type HAdV-8/D1, which was similar to the Koja strain originally isolated in Japan in 1961. The prototype strain Trim had been included under HAdV-8/D1. Five genome types (HAdV-8/D2–D6) were found among 15 strains isolated over a period of 17 years from Japan, Hungary, Austria and Germany. In this study, Adrian *et al.* (1990) created a restriction site map of HAdV-8/D1 with *Bam*HI, *Bgl*II, *Hin*dIII and *Sal*I and determined the molecular mass of the fragment. They also calculated the percentage of pairwise co-migrating restriction fragments (PCRFs) in order to measure relatedness among the genome types.

A genome typing study was carried out in Brest, France, where four consecutive outbreaks of HAdV-8 EKC occurred over a 5-year period (1983–1988). RE analysis of 30 strains isolated during this period with *Bam*HI, *Bgl*I, *Bgl*II, *Bst*EII, *Hin*dIII, *Kpn*I, *Sac*I, *Sal*I and *Sma*I revealed four new genome types, HAd-8/D7–D10. HAdV-8/D9 had also been circulating in the Netherlands in 1984 and 1988 and in Belgium in the first half of 1987. HAdV-8/D10 had also been detected in the Netherlands in 1987 (de Jong *et al.*, 1992).

During 1993–1994, nine strains of HAdV-8 were isolated from patients with conjunctivitis in the city of São Paulo, Brazil. Viral DNA RE analysis with *Bam*HI, *Bgl*I, *Bgl*II, *Hin*dIII, *Kpn*I, *Sac*I, *Sal*I, *Sma*I, *Xba*I and *Xho*I revealed two new genome types, HAdV-8/D11 and HAdV-8/D12 (Tanaka *et al.*, 2000).

Identification of HAdV-8

The conventional method of HAdV identification consists of culturing the virus and typing by NT. In the last few years, many new recombinant types of HAdVs have been identified. Some of them share the major capsid protein of HAdV-8. Therefore, instead of NT, a combination of PCR and sequencing of the hexon and fibre genes of HAdV-8 directly from a clinical sample or from isolates followed by phylogenetic analysis is recommended for molecular typing of HAdVs (Ishiko *et al.*, 2008; Ishiko & Aoki, 2009; Kaneko *et al.*, 2011).

Propagation of virus and DNA extraction

Most of the strains of HAdV-8 grow well in cells of human origin. Various continuous epithelial cell lines, such as HEp-2, HeLa, KB and A549, are useful for virus isolation, but the A549 cell line is very effective for propagation of HAdVs including HAdV-8. The cytopathic effect (CPE) of HAdV-8 in monolayer cell culture is characterized by

Table 2. Human adenovirus 8 genome type circulation: classification introduced by Adrian *et al.* (1990)

Reference	Year and country of isolation	No. of isolates	Restriction enzyme code*									Period and place of circulation	Genome type
			*Bam*HI	*Bgl*I	*Bgl*II	*Bst*EII	*Hin*dIII	*Kpn*I	*Pst*I	*Sac*I	*Sal*I	*Sma*I	
Wigand *et al.* (1983)	1983–1984, France	?	1	x	1	1	1	x	x	x	x	1		Ad8/D1
Takács *et al.* (1983)	1983, Hungary	2	1	x	x	x	1	1	1	x	1	x	1961–1962, Budapest, Szeged	Ad8/D1
Chastel *et al.* (1988)	1983–1984, France	11	1	x	1	1	1	1	x	x	1	1	1983–1984, Brest	Ad8/D1
Adrian *et al.* (1990)	1961–1982, Japan, Hungary, Austria and Germany	45	1	x	1	x	1	1	x	x	1	1	1961, Japan	Ad8/D1
		1	1	x	1	x	2	1	x	x	1	1	1961, Japan	Ad8/D2
		3	2	x	1	x	3	1	x	x	2	1	1961, Budapest	Ad8/D3
		2	3	x	1	x	4	1	x	x	1	2	1973, Vienna	Ad8/D4
		8	2	x	2	x	4	1	x	x	3	1	1977, Freiburg	Ad8/D5
		1	1	x	1	x	5	1	x	x	1	1	1977, Homburg	Ad8/D6
de Jong *et al.* (1992)	1983–1988, France	6	1	2	1	1	1	1	x	1	1	1	1983/84, 1985, Brest	Ad8/D7
		9	1	3	1	1	1	1	x	1	1	1	1984, Brest	Ad8/D8
		13	1	2	1	1	1	1	x	2	1	1	1987/88, Brest	Ad8/D9
		2	1	4	1	1	1	1	x	1	1	1	1989, Brest	Ad8/D1
Mahafzah & Landry (1994)	1984–1987, USA	18	1	x	x	x	1	x	x	x	x	1	1984, New York City; 1986, Connecticut, Ohio, New York State	Ad8/D1
Tanaka *et al.* (2000)	1993–1994, Brazil	7	1	4	3	x	1	1	x	1	3	1	1993–1994, São Paulo	Ad8/D11
		2	1	4	1	x	1	1	x	1	3	1	1993–1994, São Paulo	Ad8/D12

*x, Enzyme not used in the study.

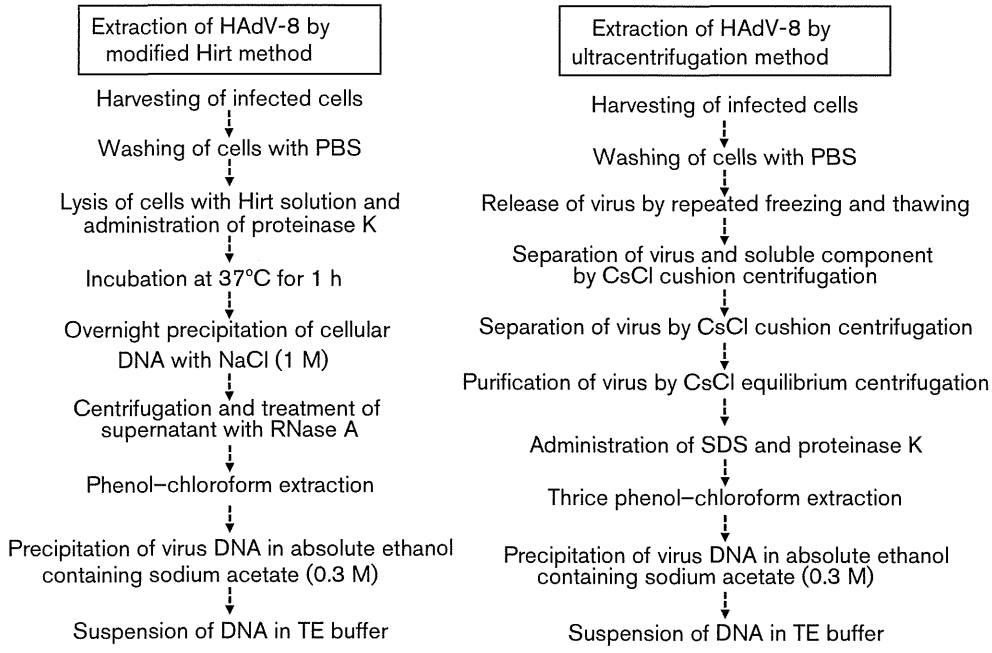

Fig. 1. HAdV-8 DNA extraction from virus-infected cells by the modified Hirt's method and by the ultracentrifugation method.

rounding and swelling of cells with nuclear enlargement followed by cellular detachment from the culture surface into grape-like clusters. However, cytological changes are not always clear by light microscopy in the case of prototype strain Trim and other fastidious strains of HAdV-8 (Guo *et al.*, 1988; Hanna & Jazetz, 1962; Wigand *et al.*, 1983; Wigand, 1987; Golden & McKee, 1970).

Viral DNA extraction from infected cells can be done by a modified Hirt's method or the ultracentrifugation method (Fig. 1) (Hirt, 1967; Wadell *et al.*, 1981; Siegel *et al.*, 1975). The modified Hirt's method of DNA extraction requires approximately 24 h and exploits the differences between low molecular mass viral DNA and high molecular mass cellular DNA. When 75–100 % CPE is visible, infected cells are dislodged and pelleted by low-speed centrifugation and cleaned with PBS. The viruses are released from the cells by mixing with lysis solution [10 mM Tris/HCl (pH 7.4), 10 mM EDTA, 1 % SDS], then the proteins in suspension are degraded by addition of proteinase K to a final concentration of 200 μg ml^{-1} at 37 °C for 1 h. After the incubation, high molecular mass cellular DNA is precipitated by addition of NaCl (5 M) to a final concentration of 1 M, and further incubated at 4 °C overnight. The suspension is then centrifuged at 15 000 ***g*** for 30 min to keep low molecular mass viral DNA in the supernatant. The supernatant is incubated with 30 μg RNase A for 1 h to remove cellular RNA and extracted twice with phenol/chloroform to separate DNA in the supernatant (aqueous phase). The supernatant is mixed in two volumes of absolute ethanol at −20 °C. Since DNA is insoluble in alcohols, it will aggregate together, giving a pellet upon centrifugation. After drying, DNA is suspended in TE buffer [10 mM Tris/HCl (pH 7.4), 10 mM EDTA] and quantified spectrophotometrically.

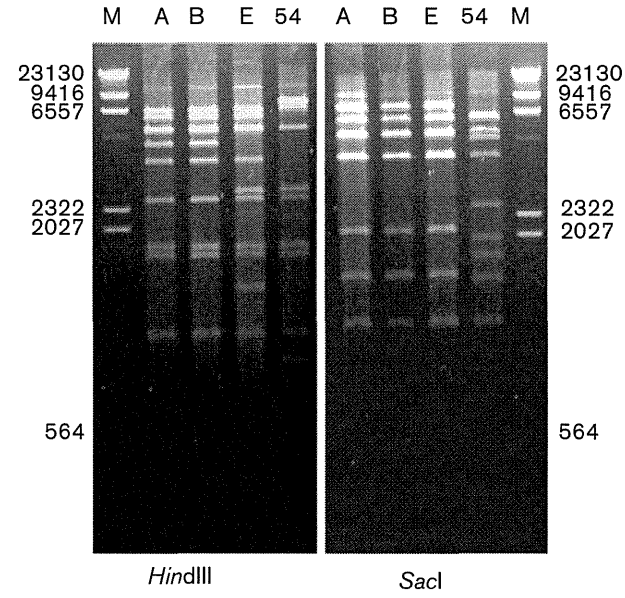

Fig. 2. Example of a 1.2 % agarose gel photograph showing restriction patterns of *Hin*dIII and *Sac*I digests of HAdV-8 genome types and HAdV-54. The *Hin*dIII digest of lambda DNA (lane M) was used as a molecular mass standard. The results in lanes A, B, E and 54 are for HAdV-8A, HAdV-8B, HAdV-8E and HAdV-54, respectively.

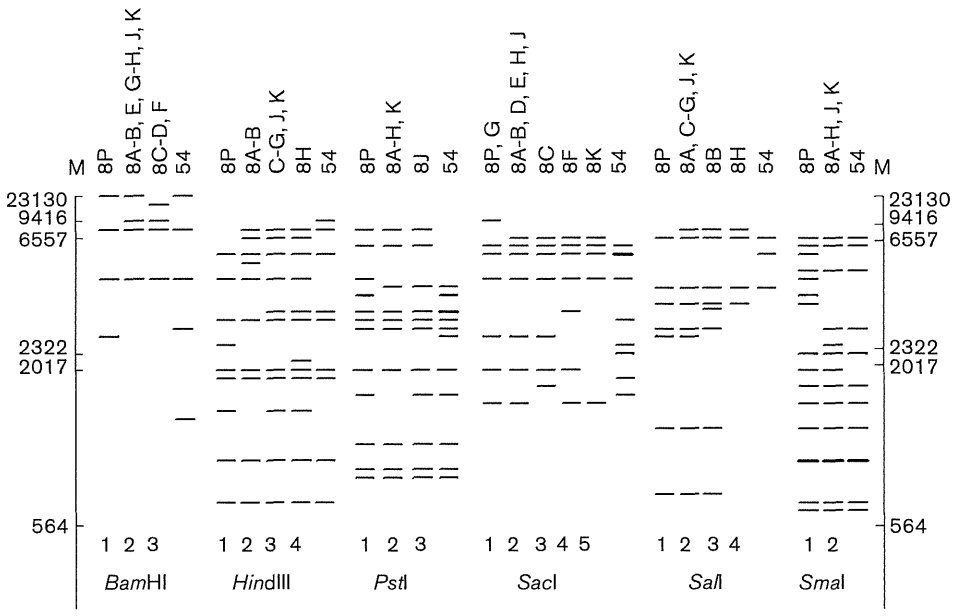

Fig. 3. Schematic presentation of RE *Bam*HI, *Hin*dIII, *Pst*I, *Sac*I, *Sal*I and *Sma*I cleavage patterns of HAdV-8A (8A), HAdV-8B (8B), HAdV-8C (8C), HAdV-8D (8D), HAdV-8E (8E), HAdV-8F (8F), HAdV-8G (8G), HAdV-8H (8H), HAdV-8J (8J), HAdV-8K (8K) and HAdV-54 (54). A *Hin*dIII digest of λ DNA (M) is used as a molecular mass standard. The restriction patterns of the HAdV-8 prototype (HAdV-8P) for each enzyme are designated number 1. The other patterns are consecutively numbered in chronological order of description of the genome type.

DNA RE analysis

This technique requires fairly large amounts of purified or partially purified DNA, and owing to small differences in the DNA of various isolates, multiple REs are required for genome typing of HAdV-8. Usually, 0.5–1.0 μg DNA is incubated with 5–10 units of RE in a 10–20 μl reaction mixture at an appropriate temperature (recommended for each RE) for 2–3 h. In addition to the clinical isolate, a reference strain is included. Reactions are stopped by addition of EDTA to a 5 mM final concentration.

The fragmented DNAs and molecular mass marker are loaded in separate wells on a horizontal submerged agarose gel and electrophoresis is done in TAE or TBE buffer. High grade and higher concentrations of gel (up to 1.2 %) yield better resolution of low molecular mass fragments (Tanaka *et al.*, 2000). DNA in the gel can be stained with ethidium bromide (1 μg ml^{-1}) after electrophoresis or the dye can be incorporated into the agarose gel during preparation. A photograph is taken under UV light (Fig. 2).

Restriction pattern analysis with schematic DNA restriction profiles and enzyme codes

Fig. 3 and Fig. 4 show a comprehensive schematic restriction profile of all genome types included in the Asian and European classification systems. The profile was created based on the published restriction patterns of HAdV-8. Enzyme codes were included in the Asian classification system in accordance with the alphabetical order of the genome types as they were described over time. The cleavage pattern of the prototype for each enzyme (*Bam*HI, *Hin*dIII, *Pst*I, *Sac*I, *Sal*I and *Sma*I) is labelled enzyme code 1. Patterns deviating from those of the prototype are labelled 2, 3, etc. Comparison of the restriction patterns of the isolates with the schematic restriction pattern could be helpful to determine the code for the respective enzyme.

Calculation of PCRFs

The genetic relatedness of HAdV-8 isolates can be calculated by using the percentage of PCRFs (Wadell *et al.*, 1980; Adhikary *et al.*, 2003, 2011; Adrian *et al.*, 1986, 1990; Kemp & Hierholzer, 1986). For example, HAdV-8A and HAdV-8B have a total of 51 co-migrating fragments and each has a total of 52 and 52 fragments, respectively. Hence the mean percentage is $(51 \times 2)/(52+52)=98$ (Table 3).

Assignment of genome type

Visual comparison of the resulting restriction patterns with the schematic restriction patterns and determination of a code for each enzyme are essential for genome typing. The distances between different digested fragments are dependent on the concentration of gel. Use of a known genome type as a reference could be helpful for comparing the results of RE analyses. If the restriction pattern of the isolate matches the schematic restriction pattern of HAdV-8, then the isolate

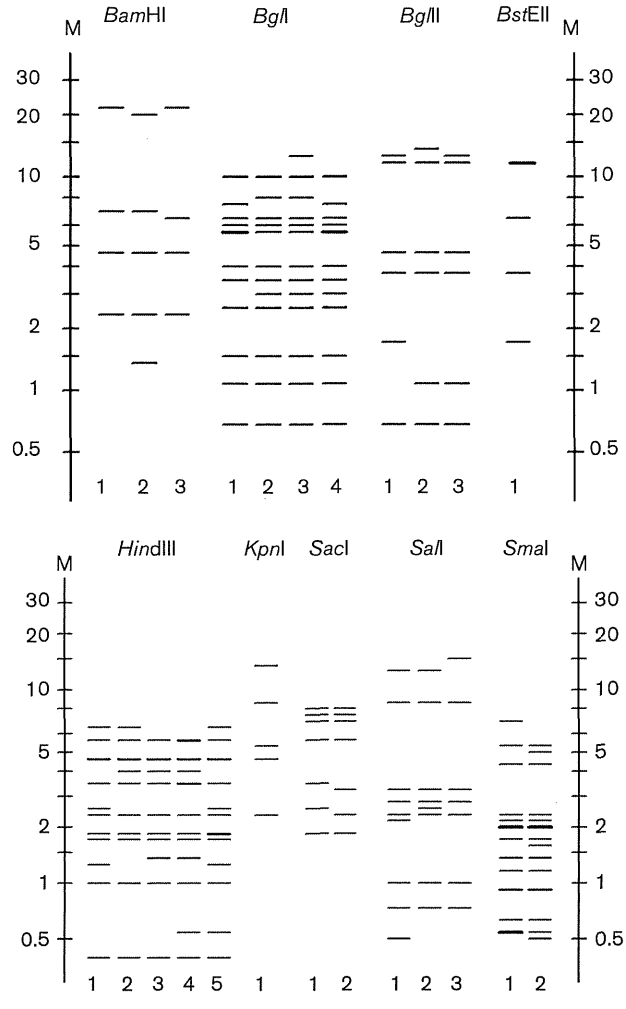

Fig. 4. Schematic presentation of RE *Bam*HI, *Bgl*I, *Bgl*II, *Bst*EII, *Hind*III, *Kpn*I, *Sac*I, *Sal*I and *Sma*I cleavage patterns of HAdV-8/D1–HAdV-8/D12. M indicates the molecular mass standard. The DNA restriction profiles of the prototype strain Trim are designated number 1 for each enzyme. Other profiles are consecutively numbered in chronological order of appearance of the corresponding DNA variant.

has an enzyme code. If the restriction patterns are new for one or more enzymes, a new enzyme code must be added. The code is to be arranged in alphabetical order of the REs as shown in the enzyme code table (Tables 4 and 5). The genome type can then be identified by comparison of the resulting enzyme codes with the enzyme code table (e.g. HAdV-8B is determined by the enzyme code arrangement 2,2,2,2,3,2 for *Bam*HI, *Hind*III, *Pst*I, *Sac*I, *Sal*I, *Sma*I, respectively). If the codes for all enzymes are already present but the arrangement (order) is different from that for previously known genome types (e.g. HAdV-8D, HAdV-8E, HAdV-8/D12), the strain can be designated a new genome type. Again, if there is a new restriction pattern for one or more enzymes, the strain will also be a new genome type.

Fig. 5 shows the schematic pathway of the genome typing from a conjunctival swab.

Discussion

EKC is not a blinding ocular infection, yet it creates distress and ocular opacity that culminates in prolonged morbidity with possible economic impact (Barnard *et al.*, 1973). Every year, HAdV-8 accounts for the highest numbers of EKC cases worldwide (Fox *et al.*, 1977; Schmitz *et al.*, 1983; Yamadera *et al.*, 1995; Kaneko *et al.*, 2011). NT is unable to differentiate one field strain from another because the viral epitopes taking part in this reaction are encoded by a small portion of the viral genome (Wadell, 1984). In contrast, RE analysis is a sensitive method for comparison of closely related genomes, second only to nucleotide sequence analysis (Wadell *et al.*, 1980). Recently, whole genome sequences became available for HAdVs, but the reported numbers of strains are still limited.

In addition, whole genome sequencing is expensive in comparison to RE analysis. Both globally dispersed and geographically restricted genome types of HAdV-8 have been identified. For example, HAdV-8D/11 and HAdV-8D/12 are constrained to Brazil and HAdV-8G and HAdV-8H to Taiwan. However, HAdV-8P and HAdV-8E are typical examples of globally dispersed strains, as they have been circulating in many countries. Co-circulation and chronological changes of genome types have also been reported in many studies. In Hiroshima, Japan, HAdV-8A and HAdV-8B co-circulated in 1983, and from 1984 to 1988, HAdV-8E co-circulated with HAdV-8A and HAdV-8B. Afterwards, HAdV-8E circulated as a single genome type until 1995 (Adhikary *et al.*, 2003). Some of these genome types seem to be associated with more severe clinical manifestations than others (Ishii *et al.*, 1987; Sheu *et al.*, 1987; Chang *et al.*, 2001). Therefore, genome typing of HAdV-8 is indispensable in epidemiological studies that offer a chance to understand the virulent nature of different strains and to follow their circulation across different geographical regions and periods of time.

RE analysis is a labour-intensive method requiring nearly 2 weeks to complete. The application of this method is challenged by many inherent problems, including (a) the fastidious nature of HAdV-8 prototype strain Trim; (b) the use of NT as a conventional method for typing of HAdV-8, which may misidentify some of the recombinant adenoviruses as HAdV-8 due to cross-reaction; (c) lack of a schematic restriction profile for all genome types collectively; (d) absence of enzyme codes in the Asian classification; and (e) use of PCRF as a tool for the estimation of genetic relatedness.

The success of genome typing depends on the extraction of a large amount of DNA from infected cells. Strain Trim and other fastidious strains of HAdV-8 grow poorly in cell culture, yielding an insufficient amount of DNA on extraction. The growth of strain Trim does not improve

Table 3. Pairwise comparison of co-migrating RE cleavage fragments from the DNA of HAdV-8 genome types

Enzyme		No. co-migrating fragments										
		P	A	B	C	D	E	F	G	H	J	K
*Bam*HI	P	4	3	3	2	2	3	2	3	3	3	3
	A		4	4	3	3	4	3	4	4	4	4
	B			4	3	3	4	3	4	4	4	4
	C				4	4	3	4	3	3	3	3
	D					4	3	4	3	3	3	3
	E						4	3	4	4	4	4
	F							4	3	3	3	3
	G								4	4	4	4
	H									4	4	4
	J										4	4
	K											4
*Hin*dIII	P	9	7	7	8	8	8	8	8	8	8	8
	A		10	10	9	9	9	9	9	9	9	9
	B			10	9	9	9	9	9	9	9	9
	C				11	11	11	11	11	11	11	11
	D					11	11	11	11	11	11	11
	E						11	11	11	11	11	11
	F							11	11	11	11	11
	G								11	11	11	11
	H									11	11	11
	J										11	11
	K											12
*Pst*I	P	12	9	9	9	9	9	9	9	9	10	9
	A		10	10	10	10	10	10	10	10	10	10
	B			10	10	10	10	10	10	10	10	10
	C				10	10	10	10	10	10	10	10
	D					10	10	10	10	10	10	10
	E						10	10	10	10	10	10
	F							10	10	10	10	10
	G								10	10	10	10
	H									10	10	10
	J										11	10
	K											10
*Sac*I	P	7	6	6	5	6	6	5	7	6	6	4
	A		7	7	6	7	7	6	7	7	7	5
	B			7	5	7	7	6	7	7	7	5
	C				7	6	6	6	6	6	6	5
	D					7	7	6	7	7	7	5
	E						7	6	7	7	7	5
	F							7	5	6	7	5
	G								6	6	6	4
	H									7	7	5
	J										7	5
	K											5
*Sal*I	P	7	7	6	7	7	7	7	7	7	7	3
	A		8	7	8	8	8	8	8	4	8	8
	B			8	7	7	7	7	7	4	7	7
	C				8	8	8	8	8	4	8	8
	D					8	8	8	8	4	8	8
	E						8	8	8	4	8	8
	F							8	8	4	8	8
	G								8	4	8	8
	H									4	4	4
	J										8	8
	K											8
*Sma*I	P	15	11	11	11	11	11	11	11	11	11	11
	A		13	13	13	13	13	13	13	13	13	13
	B			13	13	13	13	13	13	13	13	13
	C				13	13	13	13	13	13	13	13
	D					13	13	13	13	13	13	13
	E						13	13	13	13	13	13
	F							13	13	13	13	13
	G								13	13	13	13
	H									13	13	13
	J										13	13
	K											13
Total	P	54	43	42	42	42	44	42	45	44	45	38
	A		52	51	49	50	51	49	51	47	51	49
	B			52	47	49	50	48	50	47	50	48
	C				53	52	51	52	51	47	51	50
	D					53	52	52	52	48	52	50
	E						53	51	51	49	53	51
	F							53	50	47	52	50
	G								53	48	52	50
	H									49	49	47
	J										54	51
	K											52

with a change of cell lines or with a change in temperature, serum or other growth conditions (Guo *et al.*, 1988; Hanna & Jazetz, 1962; Wigand *et al.*, 1983; Golden & McKee, 1970). Moreover, repeated passage of strain Trim to obtain a sufficient amount of DNA leads to mutation of the viral genome. These properties limit the use of strain Trim as a standard in genome typing studies. HAdV-8E is included under HAdV-8/D1 and shows good growth ability (Adrian *et al.*, 1990). This genome type could be an alternative to strain Trim in RE analysis as it is widely distributed and has been circulating over a prolonged period (1983–2001).

In the last few years, many recombinant types of HAdVs have emerged as agents of EKC worldwide, including in Japan (Nakamura *et al.*, 2012). Some of these recombinant types have genomes encoding HAdV-8-like capsid proteins. HAdV-54 is one of the recombinant strains that was initially mistyped as HAdV-8 due to cross-reaction with anti-HAdV-8, and in subsequent RE analysis this strain was designated genome type HAdV-8I. Later, isolates with similar restriction patterns were identified as HAdV-54 by whole genome sequence analysis. At the present time, HAdV-54 is the common agent of EKC in many parts of Japan (Ishiko *et al.*, 2008; Ishiko & Aoki, 2009; Kaneko *et al.*, 2011). Therefore, NT may lead to spurious identification of HAdV-8, resulting in assignment of a recombinant strain as a novel genome type.

Table 4. Human adenovirus 8 genome types: Asian classification

Reference	Origin		Genome type	DNA restriction enzyme code*						No. of isolates
	Place	Year		*Bam*HI	*Hin*dIII	*Pst*I	*Sac*I	*Sal*I	*Sma*I	
Fujii *et al.* (1983)	California	1955	HAdV-8P	1	1	1	1	1	1	1
	Sapporo	1975	HAdV-8A	2	2	2	2	2	2	8
	Sapporo	1976	HAdV-8B	2	2	2	2	3	2	17
Fujii *et al.* (1984)	Kaohsiung	1980	HAdV-8C	3	3	2	3	2	2	14
	Kaohsiung	1981	HAdV-8D	3	3	2	2	2	2	2
	Kaohsiung	1980	HAdV-8E	2	3	2	2	2	2	8
	Kaohsiung	1981	HAdV-8F	3	3	2	4	2	2	3
Sheu *et al.* (1987)	Kaohsiung	1983	HAdV-8G	2	3	2	1	2	2	1
Chang *et al.* (2001)	Kaohsiung	1990	HAdV-8H	2	4	2	2	4	2	21
Adhikary *et al.* (2011)	Miyaknonojo	1998	HAdV-8J	2	3	3	2	2	2	1
Jin *et al.* (2011)	Sapporo	1991	HAdV-8K	2	3	2	5	2	2	3

*Enzyme codes are displayed in alphabetical order: *Bam*HI, *Hin*dIII, *Pst*I, *Sac*I, *Sal*I, *Sma*I. The restriction patterns of the HAdV-8 prototype (HAdV-8P) for each enzyme are designated number 1. The other patterns are consecutively numbered in alphabetical order of genome types.

Genome typing is primarily dependent on visual comparison of the resulting restriction pattern with the published restriction patterns. Because the restriction patterns of HAdV-8 field isolates are diverse, unidentified genome types of HAdV-8 have been noted in many studies (Ohguchi *et al.*, 2003; McMinn *et al.*, 1991; Mahafzah & Landry, 1994). It is not improbable that these genome types are novel or that they already existed but remained unidentified due to the lack of a catalogue of restriction profiles for all HAdV-8 genome types.

Enzyme codes were not in use in Asian classification. However, the codes for enzymes in alphabetical order and tabulated form are useful for stepwise identification of genome types. The enzyme code table helps to display the genetic variation between different genome types without

Table 5. Human adenovirus type 8 genome types: European classification

The DNA variants are denoted by numbers which are given chronologically according to the description in the literature. D1 is, by definition, the DNA variant to which prototype strain Trim belongs. The DNA restriction profiles of the prototype strain Trim are designated number 1 for each enzyme. Other profiles are consecutively numbered in chronological order of appearance of the corresponding DNA variant.

Reference	Origin		Genome type	DNA restriction enzyme code*									No. of isolates
	Place	Year		*Bam*HI	*Bgl*I	*Bgl*II	*Bst*EII	*Hin*dIII	*Kpn*I	*Sac*I	*Sal*I	*Sma*I	
Adrian *et al.* (1990)	Japan	1961	HAdV-8/D1	1	1	1	1	1	1	1	1	1	45
	Japan	1961	HAdV-8/D2	1	x	1	x	2	1	x	1	1	1
	Budapest	1961	HAdV-8/D3	2	x	1	x	3	1	x	2	1	3
	Vienna	1973	HAdV-8/D4	3	x	1	x	4	1	x	1	2	2
	Freiburg	1977	HAdV-8/D5	2	x	2	x	4	1	x	3	1	8
	Homburg	1977	HAdV-8/D6	1	x	1	x	5	1	x	1	1	1
de Jong *et al.* (1992)	Brest	1983/84, 1985	HAdV-8/D7	1	2	1	1	1	1	1	1	1	6
	Brest	1984	HAdV-8/D8	1	3	1	1	1	1	1	1	1	9
	Brest	1987/88	HAdV-8/D9	1	2	1	1	1	1	2	1	1	13
	Brest	1989	HAdV-8/D10	1	4	1	1	1	1	1	1	1	2
Tanaka *et al.* (2000)	São Paulo	1993–1994	HAdV-8/D11	1	4	3	x	1	1	1	3	1	7
	São Paulo	1993–1994	HAdV-8/D12	1	4	1	x	1	1	1	3	1	2

*x, Enzyme not used in the study.

Fig. 5. Conventional (a) and updated (b) pathway of HAdV-8 genome typing. In the updated pathway, the HAdV type can be identified directly from a conjunctival swab or infected cells by PCR-sequencing followed by phylogenetic analysis. After confirmation of HAdV-8, DNA extracted from infected cells is cleaved by multiple restriction enzymes, and genome types are assigned on the basis of the restriction pattern and code for REs.

viewing their restriction patterns, since the code for an enzyme is dependent on the restriction pattern. Therefore, the enzyme codes have been included in the Asian classification based on the alphabetical order of the genome types.

The percentage of PCRFs can be used to measure the genetic relatedness between HAdVs. The percentage of PCRFs is more than 50 % between the members of a species and less than 20 % between the members of different species (Adrian *et al.*, 1986). The percentage of PCRF is 90–98 % among the HAdV-8 genome types in Asian studies, which is higher than that observed in European studies (72 % or more) (Adhikary *et al.*, 2011; Adrian *et al.*, 1990). HAdV-54 shares a high percentage of PCRFs with other HAdV-8 genome types. Therefore, we assume that the percentage of PCRFs is useful only when the identity of HAdV-8 has already been confirmed by the molecular method.

In analysing the problems of HAdV-8 genome typing, it is evident that a simple step-by-step procedure for HAdV-8 genome typing will require (1) correct identification of HAdV-8 by molecular methods before RE analysis, (2) a schematic restriction profile for all genome types and (3) use of enzyme codes in both classification systems.

In this review, we have presented a schematic restriction profile for all HAdV-8 genome types incorporated so far in both the Asian and European classification systems by compiling all the published reports. At the same time, restriction enzyme codes for each genome type are included in the Asian classification system. We have also described a simple and organized pathway of HAdV-8 genome typing that begins with type identification and ends with interpretation of the results of RE analysis. In spite of a number of limitations, RE analysis of HAdV-8 is highly reproducible and very accurate for determining the genetic relatedness among different strains. The DNA cleavage patterns can be used as strain markers in epidemiological studies such as those evaluating nosocomial outbreaks caused by HAdV-8 (Chastel *et al.*, 1988).

Conclusions

Despite the clinical–epidemiological impact of EKC, the number of published HAdV-8 genome typing studies is lower than expected. The lack of a schematic restriction profile of all genome types as well as the need for an organized step-by-step approach have been the major obstacles to routine use of this technique. The updated system of genome typing of HAdV-8 presented here will assist public heath institutes and research laboratories to identify either existing or novel genome types so that they can be usefully compared with clinical and epidemiological data.

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