JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1986, p. 1084–1088
0095-1137/86/121084-05$02.00/0

Vol. 24, No. 6

Molecular Epidemiology of Adenovirus Type 21 in The Netherlands and the Federal Republic of Germany from 1960 to 1985

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Received 28 April 1986/Accepted 8 September 1986

After a period of high prevalence in the early 1960s, adenovirus serotype 21 (Ad21) was identified in The Netherlands only very sporadically for more than 20 years. From December 1984 to July 1985, Ad21 was isolated relatively often from hospitalized children living in different parts of The Netherlands. The patients in question suffered from respiratory, gastrointestinal, meningeal, or ocular disorders. An increase in the incidence of Ad21 infections was also observed in the Federal Republic of Germany during this period. The DNAs of 93 isolates of Ad21 were subjected to restriction enzyme analysis with eight endonucleases. All 50 strains isolated in The Netherlands between 1960 and 1963 proved to be DNA variant Ad21/D2/20655/Netherlands/60. This variant has already been described (T. Adrian, R. Wigand, and J. C. Hierholzer, Arch. Virol. 84:79–89, 1985) as typical for the Ad21 strains circulating since 1960. Analysis of the DNAs of the 28 Ad21 strains isolated in The Netherlands or in the Federal Republic of Germany in 1984 and 1985 showed them to belong to two new, closely related DNA variants designated Ad21/D7/1857/Netherlands/84 and Ad21/D8/5398/Netherlands/85. The *BglI* and *KpnI* restriction profiles were characteristic for these recent DNA variants.

Human adenoviruses are important causes of respiratory, gastrointestinal, genitourinary, and ocular diseases. Until now, 41 serotypes have been recognized in neutralization tests (7). The classification of these types into six subgenera, A through F, is based on DNA homology (10, 18), together with morphological and biological criteria (22, 24). Adenovirus type 21 (Ad21), first isolated in 1956 (4), belongs to subgenus B, which also includes Ad3, Ad7, Ad11, Ad14, Ad16, Ad34, and Ad35 (19). The members of this subgenus have recently been characterized in detail by serological methods and by DNA restriction enzyme analysis (3, 25).

In the early 1960s extensive circulation of Ad21 was observed in hospitalized children and military recruits with severe respiratory illness, both in The Netherlands (17) and in Great Britain (16). Between 1963 and 1984, more than 3,000 adenovirus isolates were typed at the Rijksinstituut voor Volksgezondheid en Milieuhygiene (RIVM), Bilthoven, The Netherlands. Ad21 was observed in only five sporadic cases. Of 148 adenovirus strains, which were collected during a period of only 7 months in 1985, Ad21 was isolated from 20 children who were not epidemiologically related. Eight isolates of Ad21 were also identified in the Federal Republic of Germany in 1985, whereas in the preceding 2 years no Ad21 strain had been isolated. Comparison of the clinical data showed that pneumonia was observed significantly more frequently during the epidemics in the early 1960s (19 of 47 patients) than during the recent outbreak in 1984 and 1985 (1 of 28 patients).

These events prompted us to explore the molecular epidemiology of this virus in The Netherlands by means of DNA restriction enzyme analysis. A short note on this work has been published (6).

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MATERIALS AND METHODS

Origin of viruses. Prototype strain Ad21 (AV-1645; strain 128, isolated in Saudi Arabia in 1956) was obtained from S. D. Bell, Cambridge, Mass. The sources of strains V1333, 8176A, 745, 1487, 20655, 21188, 630, 9794, 1213, 107782, and 122270 were given in a previous study (25).

In The Netherlands, 73 strains of Ad21 were obtained during routine diagnostic work at the RIVM between 1960 and 1985. Each strain was obtained from a different patient. The patients were living in various parts of The Netherlands and were not epidemiologically linked, except for four recruits who lived in the same army camp in 1960. Eight strains were isolated from sporadic infections in the Federal Republic of Germany during 1985, seven came from Lower Saxony, and one came from Homburg. The 81 patients in question suffered from respiratory (48 patients), gastrointestinal (23 patients), meningeal (17 patients), or ocular (5 patients) illness. The virus strains were isolated from nasopharyngeal (23 strains), fecal (56 strains), or ocular (2 strains) specimens.

All strains were typed by standard serum neutralization tests (8) with rabbit antisera to all eight adenovirus serotypes of subgenus B.

Extraction of virus DNA. Virus DNA was extracted from infected cells by a combination of the modified Hirt method (12, 21) and the procedure described by Buitenwerf et al. (5). Virus strains were grown on human diploid fibroblastic cells (strain Gabi).

DNA restriction enzyme analysis. All restriction endonucleases were from Boehringer GmbH, Mannheim, Federal Republic of Germany. Incubations were performed according to the instructions of the manufacturer. Electrophoresis was carried out at 10 V/cm at room temperature for 2 h in agarose gels (0.8% [wt/vol] agarose [SeaKem; FMC Corp., Marine Colloids Div., Rockland, Maine] in 89 mM Tris–89 mM borate–2.5 mM EDTA, pH 8.2). Gels were

TABLE 1. DNA variants of Ad21

DNA variant	Designation of:	
	Adrian et al. (3)	de Jong et al. (7)
Ad21/D1/1645/Saudi Arabia/56	Ad21 prototype	21-I
Ad21/D2/20665/Netherlands/60	Ad21′	21-II
Ad21/D3/630/Berlin/66	Ad21′	
Ad21/D4/13449/Netherlands/77		
Ad21/D5/19122/Netherlands/77	Ad21a	
Ad21/D6/107782/Hannover/80	Ad21′	
Ad21/D7/1857/Netherlands/84		21-IV
Ad21/D8/5398/Netherlands/85		21-III

stained with ethidium bromide (0.5 μg/ml) and photographed under UV illumination at 254 nm. For a few of the strains, restriction profiles carried out with four endonucleases have been reported (3). All of these patterns were in agreement with the present data except for strain V1333, which had a deviant *Hin*dIII restriction pattern, whereas the *Sma*I pattern was identical to the profile obtained for the Ad21 prototype strain.

Nomenclature of DNA variants. The designation formula for DNA variants used in this study is new: number of serotype/number of variant/designation of first identified strain of variant/geographic origin of this strain/year of isolation of this strain. For example, DNA variant Ad21′ described by Adrian et al. (3) is referred to in the present study as Ad21/D2/20655/Netherlands/60 and is abbreviated to Ad21/D2 or, if it is clear that type 21 is meant, to D2 only. The D stands for DNA, analogous to the designation H in, for example, Ad15/H9, which indicates a group of strains behaving like Ad15 in neutralization tests and like Ad9 in hemagglutination inhibition assays (1). For each serotype, the prototype DNA pattern is called D1. A complete survey on the naming of all Ad21 DNA variants (including names mentioned in previous papers) is given in Table 1.

All individual restriction enzyme patterns for the prototype DNA are labeled 1. Deviating profiles in other DNA variants are labeled 2, 3, etc. (Table 2), in the order in which they are described in the literature (2).

RESULTS

Viral DNAs of 73 Ad21 strains isolated in The Netherlands were characterized by restriction enzyme analysis. After cleavage with the restriction endonucleases *Bam*HI, *Bgl*I, *Bgl*II, *Bst*EII, *Hin*dIII, *Kpn*I, *Sma*I, and *Xho*I, five DNA variants were observed, each different from the Ad21 prototype (Table 3). The restriction fragment profiles of representatives of these DNA variants are shown in Fig. 1.

Identical restriction enzyme patterns were observed for all 48 Ad21 strains which were collected at the RIVM during the years 1960 to 1963 and also for two strains, 20655 and 21188, which were isolated during a screening of Dutch military recruits in 1960 (17). This DNA variant was designated Ad21/D2/20655/Netherlands/60 according to the rules for naming DNA variants described in Materials and Methods. Two of the five isolates from the interepidemic period from 1963 to 1984 also were DNA variant D2, while two other isolates belonged to DNA variants Ad21/D4/13449/Netherlands/77 and Ad21/D5/19122/Netherlands/77. Characteristic for variants D4 and D5 is the deviant *Hin*dIII profile, which lacks one of the two second largest fragments and contains two new fragments: a small fragment at the bottom of the gel and a fragment of about the same size as the fourth largest fragment (Fig. 1). The fifth strain of Ad21 isolated between 1963 and 1980 (isolate 76-5328) could not be classified because the DNA characterization of this strain was prevented by contaminating DNA of a yet unknown nature.

TABLE 2. Restriction profiles of Ad21 DNA variants

DNA variant	Profile obtained with:							
	*Bam*HI	*Bgl*I	*Bgl*II	*Bst*EII	*Hin*dIII	*Kpn*I	*Sma*I	*Xho*I
D1	1	1	1	1	1	1	1	1
D2	2	2	1	2	1	2	2	2
D3	2	2	1	2	1	1	2	2
D4	1	2	1	1	2	3	1	2
D5	1	2	1	1	2	1	1	2
D6	2	2	1	3	1	1	2	2
D7	3	3	1	2	1	4	2	2
D8	2	3	1	2	1	4	2	2

Twenty strains were isolated in The Netherlands during the 1984-1985 season. The DNAs of these strains belonged to two new DNA variants, Ad21/D7/1857/Netherlands/84 and Ad21/D8/5398/Netherlands/85. Most characteristic for the new variants were the clearly deviant *Kpn*I profile (Fig. 1) and the *Bgl*I pattern, which shows the lack of the smallest *Bgl*I fragment and a slightly larger *Bgl*I fragment at the top of the gel. Among the restriction enzymes which were used in the present study, only *Bam*HI could discriminate between variants D7 and D8. The DNAs of the eight recent Ad21 isolates from the Federal Republic of Germany proved to be variant D8.

In a previous paper on DNA analysis of the Ad21 serotype, several strains isolated in the Federal Republic of Germany and the United States between 1966 and 1982 have been described (3). By analysis with the endonucleases *Sma*I, *Bam*HI, *Bgl*II, and *Hin*dIII, these strains were shown to be identical and were provisionally designated Ad21′ (Table 1). The DNA restriction analysis of nine of these strains was extended in the present study by the additional use of four other enzymes (Table 3). The analyses revealed that the five strains from the Federal Republic of Germany belonged to two new DNA variants, Ad21/D3/630/Berlin/66 and Ad21/D6/107882/Hannover/80, which differ from each other only in their *Bst*EII profiles (Fig. 2). Strains 8176A, 745, and 1487 from the United States (11) exhibited patterns identical to that of DNA variant D2, whereas strain V1333 (Ad21a in reference 3) had DNA characteristic for variant D5.

After demonstrating the existence of a number of DNA variants, we examined whether these variants reacted differently in neutralization tests. The infectious dose in these tests was made equal for all virus preparations by using that dilution which produced 100% cytopathic effect in about 7 days (experiment A). The titers of rabbit anti-Ad21 antiserum against the various strains varied widely indeed, but no systematic differences among the DNA variants could be discerned (Table 4). The best correlate to the magnitude of the serum titer was the magnitude of the virus dilution: the higher the virus dilution, the higher the serum titer. This suggests that at low dilutions a large part of the antibodies was consumed by the high antigenic load. In these cases, new titrations were performed with virus dilutions of at least

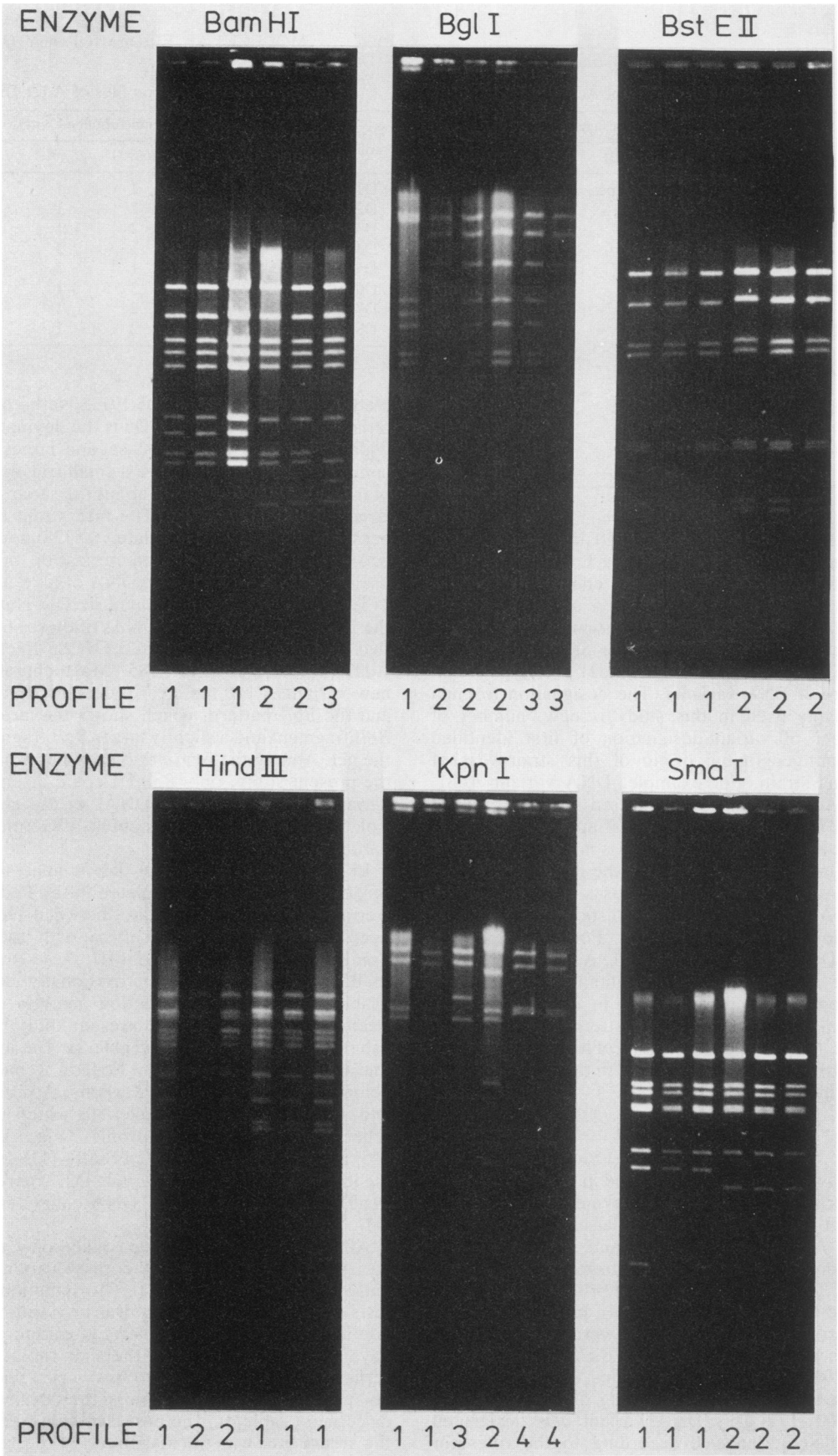

FIG. 1. Restriction enzyme analysis of Ad21 strains. Restriction profiles prepared by using six enzymes are shown for the Ad21 prototype strain and five Ad21 strains, each representing one of the five DNA variants of Ad21 isolated in The Netherlands. In each panel the strains are depicted in the same order: Ad21 prototype strain (DNA variant D1), strain 19122 (DNA variant D5), strain 13449 (DNA variant D4), strain 20655 (DNA variant D2), strain 5398 (DNA variant D8), and strain 1857 (DNA variant D7).

TABLE 3. Molecular epidemiology of Ad21

Strain origin (no. of strains) and DNA variant	No. of strains isolated in:															
	1956	1960	1961	1962	1963	1966	1973	1976	1977	1978	1979	1980	1981	1982	1984	1985
Saudi Arabia (1), D1	1															
The Netherlands (75)[a]																
D2		6	30	2	10		1					1				
D4									1							
D5									1							
D7															1	12
D8																7
Unknown								1								
D2[b]	2															
Federal Republic of Germany (13)																
D3						2					1			1		
D6												1				
D8																8
United States (4)																
D2								1	1	1						
D5													1			

[a] Including all 73 Ad21 strains isolated and typed between 1960 and 1985 at the RIVM.
[b] Two strains, 20655 and 21188, were isolated during a screening of Dutch recruits in 1960 (17).

1:200 and by using an additional virus passage if necessary. Under these conditions (experiment B), the resulting antiserum titers with all DNA variants tested were more similar (Table 4).

All strains of Ad21 studied here displayed weak neutralization by anti-Ad35 antiserum at routine dilution, a finding which is in agreement with previous reports (11, 25). The titer ratio of anti-Ad21 to anti-Ad35 did not vary significantly among the various DNA variants (data not shown). In conclusion, the results of the neutralization tests did not indicate that the DNA variants differed strongly in their serological reactivity.

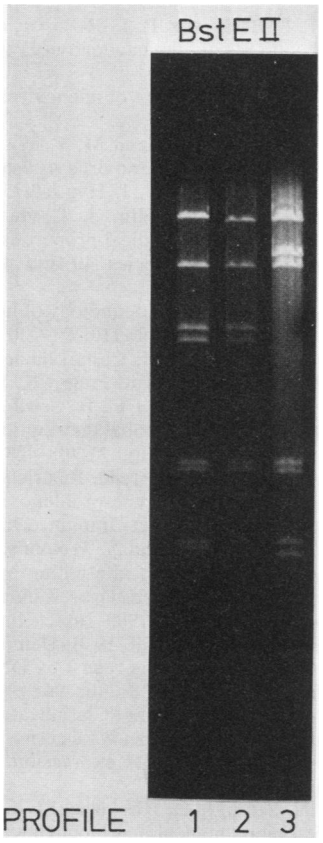

FIG. 2. DNA restriction enzyme analysis of Ad21 with *Bst*EII, showing (from left to right) Ad21 prototype DNA (profile 1), strain 20655 (profile 2), and strain 107782 (profile 3).

DISCUSSION

The mutation rate during replication is much lower with DNA viruses than with RNA viruses (13). Yet the heterogeneity of the adenoviruses is considerable, as reflected in 41 serotypes and in differences in hemagglutination patterns among strains of one serotype, e.g., Ad15/H9 (1). The heterogeneity of the DNA of adenovirus strains is most clearly demonstrated by DNA restriction enzyme analysis. DNA variants have been described for various serotypes, e.g., Ad7 (23), Ad19 (21), Ad40, Ad41 (14, 15), and many others (24). The DNA of Ad7 has a particular variation in time. A shift from DNA variant Ad7c to Ad7b was observed in 1969 in Europe and in 1975 in Australia (20, 22).

The present study describes a similar history of Ad21 in The Netherlands. Each of the three distinct epidemiological periods of Ad21—namely, 1960 to 1963, 1964 to 1983, and 1984 to 1985—proved to be associated with different DNA variants (Table 3). During the epidemic phase of 1960 to 1963, only variant D2 was found among 50 isolates. From 1964 to 1983, only five strains were isolated, representing at least three variants: D2, D4, and D5. In this period, variants D2 and D5 were isolated also in the United States (3, 11, 25). Finally, the epidemic of 1984 and 1985 was caused by two new cocirculating variants, D7 and D8. Variant D8 was also isolated from eight patients in the Federal Republic of Germany in 1985.

Ad21 infections were significantly more associated with pneumonia in the period from 1960 to 1963 than they were during the recent outbreak. Whether or not this observation indeed reflects a difference in pathogenicity between variant D2 and variants D7 and D8 is difficult to determine. We can also only speculate about the cause of the revival of Ad21 in The Netherlands after a dormancy of 21 years. The continuous presence of variant D2 during the quiet period, as evidenced by two isolates from The Netherlands and three

TABLE 4. Neutralization tests comparing various DNA variants of Ad21[a]

Ad21 strain	DNA variant	Virus dilution	Days of incubation[b]	Neutralization titer[c]
Expt A				
Prototype	D1	1:200	7	3,072
60-2485	D2	1:2	7	96
76-8176A	D2	1:2	7	32
80-12506	D2	1:30	7	768
77-13449	D4	1:3,000	6	4,096
81-V1333	D5	1:2,000	6	2,048
85-1857	D7	1:300	7	3,072
85-9132	D7	1:30	9	192
85-11172	D7	1:1,000	6	6,144
85-5398	D8	1:3	7	48
Expt B				
Prototype	D1	1:200	7	3,072
60-2485	D2	1:200	17	1,024
76-8176A	D2	1:200	16	768
77-13449	D4	1:3,000	6	4,096
81-V1333	D5	1:2,000	6	2,048
85-1857	D7	1:300	7	3,072
85-9132	D7	1:3,000	20	4,096
85-11172	D7	1:1,000	6	6,144
85-5398	D8	1:300	14	768

[a] Neutralization tests were performed either with equal infectious doses for all virus preparations (experiment A) or with viral dilutions of at least 1:200 (experiment B).

[b] After virus inoculation, the Gabi cell cultures were incubated until 100% of the cells showed adenovirus cytopathic effect.

[c] Neutralization titers of antiserum to Ad21 prototype were read as the reversed antiserum dilution which reduced the cytopathic effect to <25% of the cells.

isolates from the United States, indicates that after 1963 solid group immunity was probably built up, preventing a high incidence of Ad21 infection. High titers of neutralizing antibodies against Ad21 have indeed been found in pooled human gamma globulin preparations (9). The change in environmental epidemiological conditions might also have played a role (e.g., the decreased crowding of military recruits) (17). In any case, before Ad21 again initiated a significant circulation, it had bred two new DNA variants with more favorable antigenic determinants or with other factors enhancing its spread among humans. Indeed, we found some differences in antigenic reactivity among the variants (Table 4). Monoclonal antibodies may bring out even larger differences, as they did among DNA variants of Ad40 and Ad41 (H. G. A. M. van der Avoort and J. C. de Jong, manuscript in preparation). Our data show clearly, however, that over a period of 30 years Ad21 was not involved in anything like the "antigenic drift" which enables influenza virus to return almost yearly.

ACKNOWLEDGMENTS

We thank J. G. Kapsenberg for advice about the manuscript; B. Postma, A. Dumas, M. Lambriex, and K. W. Knocke for providing the Ad21 strains; and G. Schäfer for technical assistance.

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