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Genome Type Analysis of Human Adenoviruses Associated with Pediatric Gastroenteritis in Korea

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=국문초록=

소아장염을 유발하는 한국형 아데노바이러스들의 지놈형별

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김희진[1] · 양재명[1] · 김경희

대략 36,000 base pairs (bp)의 두 가닥짜리 DNA를 지놈으로 가진 사람 아데노바이러스 (Ad)는 DNA 相同性 및 생물학적/생화학적 성격이 특이한 49개의 혈청형이 알려져 있는데, 이들 대부분의 Ad가 영유아군 및 면역능이 저하된 성인에서 치사적 결과를 초래할 수 있다. Ad의 세포向性 (tropism)은 매우 다양하여 종류에 따라 상기도 감염, 각결막염, 영유아 장염등을 유발하는데 최근 Ad의 다양한 병원성에 대한 원인을 분자생물학적 수준에서 규명하려는 노력의 일환으로 지역에 따라 주되게 출현하는 Ad형 규명이 활발히 이루어지고 있다. Ad동정/확인은 표면을 이루고 있는 group 공통항원인 hexon 단백질을 탐지하는 효소면역측정법 (EIA)에 의하며, Ad형별은 Ad fiber의 세포독성 중화시험에 의한다. 그러나, 세포독성 중화시험이 엄청난 노동력 및 시간을 요구하면서도 민감도/특이도가 만족스럽지 못하여 이를 개선하기 위하여 검체 또는 세포배양에서 Ad DNA를 추출하여 제한효소 절단형태를 비교하는 방법이 개발되었는데 이는 세포배양에 잘 자라지 않는 바이러스주의 형별뿐만 아니라 지역 분리주들의 지놈 변형주를 관찰하는 분자생물학적/분자역학적 연구에도 도움이 되고 있다.

국내에는 Ad와 관련된 소아장염의 빈도가 rotavirus에 의한 것 다음으로 빈번한데도 Ad40/41외에 주되게 출현하는 장내 Ad형들이 전혀 규명된 바 없고, 한국형 Ad들의 지놈형태가 전혀 보고된 바가 없다. 또한 세계적으로 Ad형별 조사지역이 늘어감에 따라 유아장염과 연관된 Ad 역시 Ad40, 41이외의 형들이 Ad40, 41을 능가하는 것으로 보고되고 있는 지역도 있으나 국내에서는 Ad40, 41이외의 형들은 그 역학적 중요도가 전혀 알려져 있지 않다. 이로서 본 연구의 목적은 Ad주들에 특이 중화항체를 이용한 세포독성 중화시험과 Ad DNA 절단법을 적용하여 한국형 장내 Ad주들의 형별을 처음으로 시도함과 동시에 1989-1991사이 출현한 Ad들의 유전적 변형을 관찰하려는 것이었다.

두 방법 모두 사용하였을 때 주되게 출현하는 장내 Ad형들은 Ad41, Ad2, Ad7, Ad5, 및 Ad40이었다. Ad40/41-양성 검체를 제외한 Ad hexon-EIA 양성들의 77.5%를 형별할 수 있었던 Ad DNA의 제한효소 절단방법은 형들간의 교차중화로 특이성이 낮았던 중화방법 (47.5%)보다 매우 효율적이어서 두 가지 방법을 함께 적용하였을때는 40주중의 87.5%인 35주를 형별할 수 있었다. 또한 Ad DNA 제한효소 절단방법은 Ad7 변이주 (Ad7b)도 탐지할 수 있었다.

Key Words: Human adenovirus, Gastroenteritis, Adenovirus types, DNA restriction analysis

INTRODUCTION

Although human adenoviruses (Ad) cause a variety of diseases such as acute respiratory, ocular, gastrointestinal, and urinary tract infections in both adults and children, they are most commonly isolated from immunosuppressed patients and from children with lower respiratory or gastrointestinal illnesses [1-6]. Until now, 49 serotypes of human Ad have been described [1,7].

Given the importance of Ad as etiologic agents of pediatric gastroenteritis [8-13], a number of assays have recently been developed for the direct detection of Ad in stool specimens. Traditionally, typing of Ad has been performed by reacting defined antisera (made against prototype viruses) with the isolate to be typed in a neutralization assay, accepted by the International Committee on Taxonomy of Viruses as the definition of an Ad type [6,14]. However, neutralization assays are time-consuming, sometimes difficult to interpret due to cross-reactivity, and absolutely dependent upon the availability and quality of type-specific antisera. In an effort to improve the speed and accuracy of Ad typing, immunoassays designed for detecting Ad group-specific antigen (hexon) and for detecting enteric types, Ad40/41, that accounted for the majority of cases of Ad gastroenteritis [9-13] were developed [2,4]. Moreover, restriction endonuclease digestion of Ad DNA has been adapted to distinguish between serotypes of human Ad prototypes 1 to 41, and many genome types have been recognized in Ad strains within the same serotype [6,15]. DNA analysis by restriction endonucleases has turned out to be a powerful method also to characterize noncultivable viruses and to trace infection chains [16].

In Korea, Ad are considered to be second only to rotaviruses as the most significant cause of gastroenteritis in young children [9,10] and thus it is essential to know the full spectrum of Ad serotypes and their genomic profiles routinely present in stool specimens from symptomatic patients. The study reported here therefore had two objectives: (1) to evaluate the genetic variability of Korean Ad strains associated with pediatric gastroenteritis, and (2) to compare DNA restriction analysis and serum neutralization methods for typing Ad isolates from stool specimens. A retrospective analysis was therefore done with 40 Ad-positive stool specimens collected from 1989 to 1991.

MATERIALS AND METHODS

1. Collection of specimens

The 61 stool specimens used in this study were obtained during 1989 to 1991 and were from children with diarrhea and were identified as Ad-positive by enzyme immunoassay (EIA) with a monoclonal antibody to the hexon antigen of Ad [9,10]. All specimens were stored at -70℃ prior to inoculation of cell cultures for virus isolation.

2. Virus isolation

Fifty-nine viral isolates were obtained by inoculation of Ad-positive stool specimens into duplicate tubes of either HEp-2 cells (American Type Culture Collection CCL23 [epidermoid carcinoma, larynx, human]) or Graham 293 cell (ATCC CRL1573 [transformed primary embroynal kidney, human]). The cells were grown in Eagle's minimal essential medium (EMEM) with 10% fetal bovine serum (FBS) and were subcultured with a trypsin-EDTA solution [2,14].

Ad-positive stool specimens were diluted 10-fold in Hank's balanced salt solution (HBSS) containing penicillin-streptomycin-amphotericin B and 5% chloroform, incubated 10 min in a shaking incubator, clarified by centrifugation in an eppendorf centrifuge at 12,000 × g for 15 min, and filtered thru 0.45μm filter membrane.

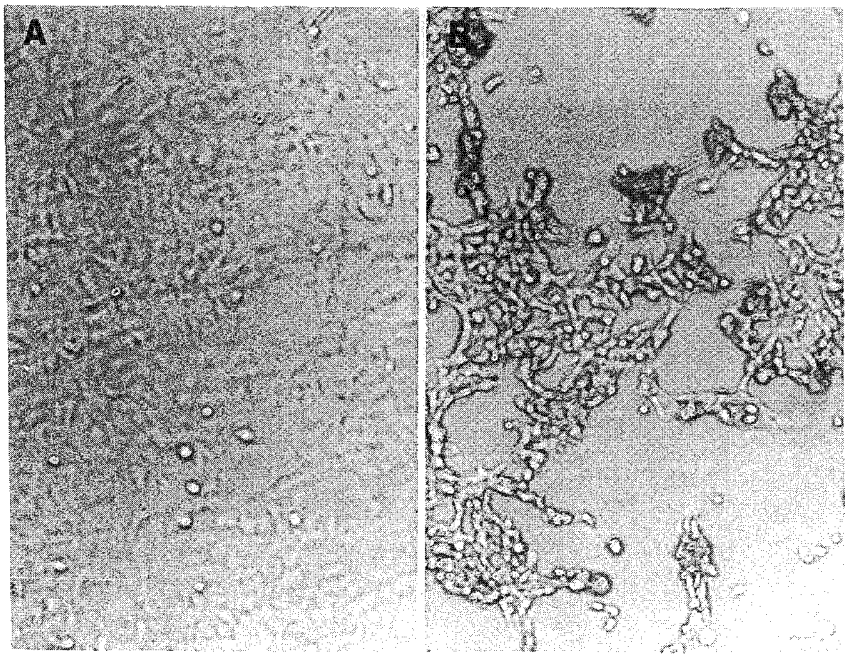

Figure 1. HEp-2 cell monolayers. A shows a normal appearance and B exhibits a 3+ or greater viral cytopathic effect. Cells in tightly associated grapelike clusters are observed.

Two hundred mls of supernatants were inoculated onto the first duplicate subconfluent cultures in 96-well microplates (Flow Laboratories, McLean, VA) containing 1 ml of EMEM with 2% FBS and serially titrated by adding 200 mls of the first duplicate wells to the second duplicate wells and so on up to the 6th duplicate wells. The plates were incubated in a 5% CO_2 environment at 35℃. Ad was identified by typical cytopathic effect within 2 to 7 days postinoculation and confirmed by Ad hexon antigen EIA [2,14](Figure 1). Viral stocks of each of the Ad isolates were stored at -70℃ for typing of the progeny virus by microneutralization and for extraction of the viral DNA for restriction endonuclease analysis.

3. Serotyping of virus isolation

For typing by microneutralization, Ad stocks were grown in HEp-2 cells and titers were determined in flat-bottom 96-well plates containing confluent HEp-2 cells. For the neutralization assay, 100 tissue culture infectious doses of each stock virus were incubated with 0.025 ml of antiserum to adenovirus types 1-3, 5-7, 12, 25, 31, 34, 35, 40, and 41 obtained from either the Centers for Disease Control, Atlanta, Ga., or the American Type Culture Collection, Rockville, Md. Virus isolates were tested first against antisera specific for Ad31, Ad40, and Ad41, since they were suspected of the most common types, and if they were not completely neutralized, they were then tested individually against the other antisera. The Ad type was determined by the antiserum, which completely inhibited viral growth after 72 h of incubation [2,14]. Isolates which exhibited neutralization with more than one antiserum were reassayed. Isolates which were neutralizaed by more than one antiserum on repeated assays were designated as nontypable.

4. DNA extraction

Cells were observed for cytopathic changes;

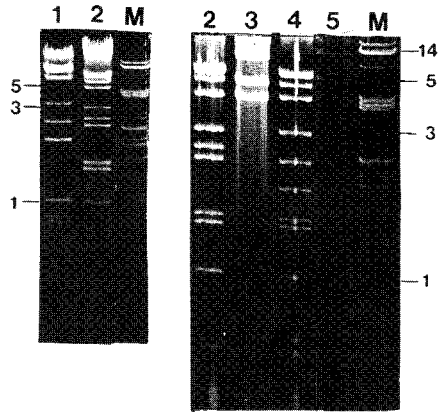

Figure 2. Analysis of Adenoviruses by restriction mapping. Six isolates were analyzed by digestion with *Sma*1. Lane 2 was Ad serotype 1 ro 2. Lanes 1, 3, 4 and 5 were types 34, 31, 41 and 7, respectively. Marker (M) was λ DNA digested with *Hin*dIII-*Pst*I.

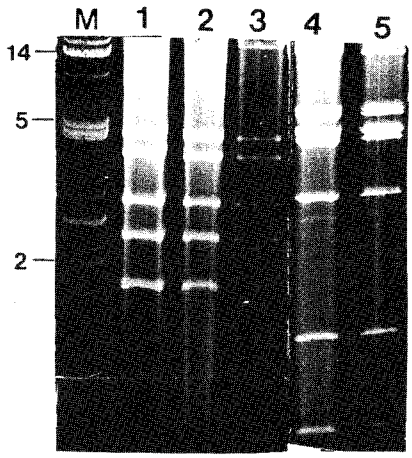

Figure 3. The Ad1/2 were analyzed by digestion with *Eco*R1 and two Ad3/7 by *Bam*H1. Lanes 1 through 3 were Ad serotype 2. Lanes 4 and 5 were types 7 and 7b, Marker (M) was λ DNA digested with *Hind*III-*Pst*I.

when extensive cytopathic change was evident, DNA was extracted by a modification of the Shingawa procedure [17]. Two hundred mls of cells and media were lysed by the addition of 5x lysis buffer (50mM Tris [pH7.8], 2.5% sodium dodecyl sulfate, 24 mM EDTA) 100 μl, and proteinase K (10mg/ml) 25 μl and incubated at 52℃ for 1 hr. The viral DNA was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1, vol/vol) and twice with chloroform:isoamyl alcohol (24:1). DNA was concentrated by precipitation with 2 volumes of ethanol and sodium acetate with a final concentration of 0.5 M at -20℃ overnight.

5. Restriction enzyme analysis of viral DNA

The total size of the DNA is about 36,000 bps. Aliquots containing 1 to 2 μg of viral DNA were digested with 10 to 15 U of different endonucleases and type assignments were made by comparing the resulting pattern with patterns of prototype viruses [15,16]. All restriction endonucleases were purchased from Boehringer-Mannheim Ltd. (Gaithersburg, MD), and digestions were carried out under conditions specified by the supplier. After digestion, samples were loaded onto 0.8% agarose gels and electrophoresed at 80 V for 3 hr in a 50 mM Tris-borate-EDTA buffer [17]. After staining with ethidium bromide (0.5 ug/ml), the DNA bands in the gels were photographed with a UV transilluminator. Some typical patterns are shown in Fig. 2 and 3. For adenovirus types in which the *Sma*I patterns were identical (e.g., Ad1/Ad2, and Ad3/Ad7), additional digestions with *Eco*R1 for Ad1 and Ad2 and with *Bam*H1 for Ad 3 and 7 were performed (Fig. 4). *Hind*III restriction analysis was then used to distinguish circulating strains of both Ad40 and Ad41 strains.

RESULTS

1. Ad isolations

In the present study, designed to evaluate the genetic variability of Ad strains associated with pediatric cases of acute gastroenteritis for the first time in Korea, a collection of 61 Ad hexon EIA-positive specimens were inoculated into HEp-2 cells and/or Graham 293 cells. Of the 61 Ad isolates, 3 isolates grew very poorly and could not be further analyzed; thus the

Table 1. Method Comparison

DNA result (Adenovirus subgenus and type)		No. of isolates by neutralization analysis for following adenovirus subgenus and type									
		A	B			C			D	M[a]	NT[b]
		31	3	7	34	1	2	5	25		
A	31	1									2
B	3		1								
	7[c]			1							5
	34				1						2
C	1					3					1
	2						7				5
	5								1		6
D	25								1		
NT											1
No DNA			1				2			1	4

[a] No DNA, but neutralized by multiple antisera.
[b] NT, Not typable.
[c] Includes variants with slightly altered cleavage patterns (genome types).

rest 59 (96.7%) isolates were characterized by both restriction enzyme analysis and neutralization assays.

2. Ad serotyping by microneutralization

Of the 59 tissue culture-grown isolates, only 20 (33.9%) isolates could be assigned to a type by microneutralization. For 5 of these 20 isolates (25%), initial testing demonstrated partial neutralization with more than one adenovirus anti-serum, and repeat testing was needed to ascertain which type-specific antiserum gave the most reproducible endpoint. Ad40 and Ad41 exhibited extensive cross-reactivity in virus neutralization. Overall, 39 isolates could not be definitvely assigned by microneutralization testing to a single Ad type.

Of the 20 isolates that were assigned to the particular type, 9 (45%) isolates were serotype 2, 3 isolates were serotype 1, 2 isolates were serotype 3, and another 2 isolates were serotype 25. The remaining serotypes included Ad 31, Ad7, and Ad34.

3. Genomic analysis

Fifty-nine isolates were subjected to restriction endonuclease digestion of viral DNA. The total number of restriction fragments was 40 or more, except for Ad31. Since Ad31 has only 5 fragments upon *Sma*I digestion, any isolate showing Ad31-like pattern, were redigested with *Msp*I (Fig. 4). There was a high degree of comigration in pairwise comparisons of Ad1, 2, and 5 with 59 to 77% comigrating fragments.

Of the 59 isolates, 49 (83.1%) could be clearly assigned a type. Eleven (22.4%) were Ad41, 10 (20.4%) isolates were Ad2, 6 (12.2%) isolates were Ad7 (5 Ad7b), 5 (10.2%) were Ad5, and 4 (8.2%) were Ad40 which together accounted for almost 74% of the isolates. The remaining virus isolates were typed as Ad1, 31, 34, 3, and 25. In addition, 5 isolates which were not typable by neutralization were found to be genome type 7b.

4. Comparison of the two methods

A total of 53 (89.8%) isolates were typed by both restriction enzyme analysis and neutralization assays.: the predominant types were Ad41, Ad2, Ad7 (including Ad7b), Ad5, and Ad40. The incidence of Ad31 or Ad3 was relatively insignificant. DNA restriction analysis (83.1%) proved to be much better than serum neutralization partially because DNA restriction enzyme patterns distinguish between

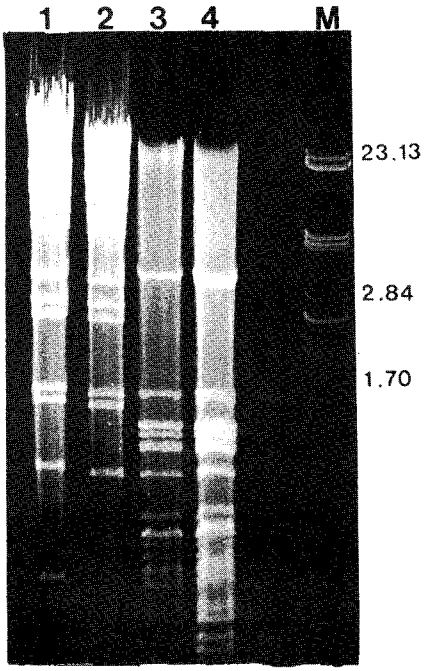

Figure 4. Analysis of Adenoviruses by restriction mapping. Two isolates were analyzed by digestion with *Sma*I (lanes 1 and 2 are Ad serotype 1 ro 2) and another with *Msp*I (lanes 3 and 4 are Ad31). Marker (M) was λ DNA digested with *Hind*III-*Pst*I.

Ad40 and Ad41.

Excluding isolates with Ad40/41 specificity, restriction enzyme analysis was able to determine 77.5% of EIA-positive samples while microneutralization permitted to type only 47.5% of the same samples. Table 1 shows the results of the two methods. In addition, the DNA analysis method allowed the identification of genomic variants of Ad7. Typing clinical isolates of Ad by restriction endonuclease digestion of viral DNA was done rapidly, provided additional epidemiological and typing information, and provided fewer ambiguous results than did typing by neutralization.

DISCUSSION

As demonstrated in recent reports [9,10] in Korea, Ad has been the second most frequently detected virus in children hospitalized for acute gastroenteritis, accounting for around 9 to 12% of admissions but molecular epidemiology of Ad gastrointestinal infections in Korea is still poorly characterized. Therefore, studies aimed at the analysis of circulating Ad strains are necessary. We present the results of the genome typing of Ad strains isolated from 61 stools collected from children hospitalized for gastroenteritis at the Hanyang University Pediatric Hospital, Seoul, Korea between 1989 and 1991 as part of an ongoing surveillance program for enteric viral pathogens.

In this series of 53 clinical Ad isolates, Ad typing by DNA analysis appeared to be more accurate and provided more information than standard microneutralization methods. Using the microneutralization assay, we identified 20 isolates which included 7 different Ad types. Of the typable isolates 5 (25%) had microneutralization assay results that required repeating one or more times to determine the Ad types definitely. Of the 53 isolates, 14 (26.4%) were not typable by microneutralization testing, demonstrating partial neutralization with several antisera even on repeat analysis. With DNA analysis, 49 isolates were typed, and these included 10 different Ad types. The predominant types were Ad41, Ad2, Ad7 (including Ad7b), Ad5, and Ad40. The incidence of Ad31 or Ad3 in this study was relatively insignificant. In Japan [19], Canada [20], and Sweden [21], Ad41, Ad31, and Ad40 together accounted for the majority of the specimens.

Apart from the fact that neutralization tests take considerably more time than DNA restriction analysis, we had difficulty in distinguishing Ad40 and Ad41 by neutralization tests due to extensive cross reactivity as reported in an earlier study by de Jong *et. al.* [22]. Another advantage of the DNA analysis technique was that it permited identification of new types, Ad7b. Ad7b of subgenus B cir-

culated over the study period sporadically, and was the predominant Ad7 genome type. The occurrence of an Ad7 genome type shift has been recorded in the former Soviet Union [23], from Ad7a to Ad7f. Since there has been any baseline data in Korea, it is noteworthy to continuously observe the currently predominant genome types circulating. Among the factors that may account for the substitution of a genomic variant by another, however, a greater capacity to spread and a higher virulence should be considered, although differences in pathogenicity have been difficult to prove.

CONCLUSIONS

A total of 53 (89.8%) isolates were typed by both restriction enzyme analysis and neutralization assays.: the predominant types were Ad41, Ad2, Ad7 (including Ad7b), Ad5, and Ad40. The incidence of Ad31 or Ad3 was relatively insignificant. Excluding isolates with Ad40 or Ad41 specificity, restriction enzyme analysis was able to determine 77.5% of EIA-positive samples while microneutralization permitted to type only 47.5% of the same samples.

Although Ad40 and Ad41 exhibit extensive cross-reactivity in virus neutralization assays, they were defined as separate types on the basis of major differences in DNA restriction enzyme patterns. In addition, the DNA analysis method allowed the identification of genomic variants of Ad7. Typing clinical isolates of Ad by restriction endonuclease digestion of viral DNA was done rapidly, provided additional epidemiological and typing information, and provided fewer ambiguous results than did typing by neutralization.

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