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Rapid Identification of Human Adenovirus Types 3 and 7 from Respiratory Specimens via Multiplex Type-Specific PCR

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The rapid diagnosis of human adenovirus (Ad) infection is crucial for the timely recognition of epidemics. Moreover, identification of the serotypes known to cause serious disease can be helpful in therapeutic intervention. A multiplex PCR assay was developed for the rapid detection of adenovirus type 3 (Ad3) and Ad7 directly from clinical specimens. For this assay, three primer pairs (primers were based on the conserved and hypervariable regions of the hexon) were designed in order to simultaneously amplify all adenoviral serotypes and discriminate between Ad3 and Ad7. In our preliminary analysis, this multiplex PCR assay generated amplicons of the consensus primers from all 106 adenoviral isolates of diverse serotypes and proved able to correctly identify Ad3 and Ad7. This assay was subsequently applied to the detection of Ad3 and Ad7 in respiratory specimens. Among the 127 nasal aspirates from which an adenovirus was grown, the sensitivity with which any serotype could be detected was 91% (115/127). Two of the 53 nasal aspirates which did not grow Ads yielded adenovirus-specific bands, which were confirmed by sequencing analysis. Among the 115 specimens which produced common adenoviral bands, the sensitivity with which Ad3 could be detected was 93% (26/28), and the sensitivity with which Ad7 could be detected was 100% (35/35). Five out of the 115 specimens were proved to harbor more than one type of Ad via sequencing analysis of the amplicons, suggesting mixed infection with at least two different serotypes. In conclusion, this multiplex PCR system can be utilized in the rapid identification of Ad3 and Ad7 directly from clinical specimens. Furthermore, this method constitutes a diagnostic strategy for the detection of coinfection by different Ad serotypes.

Human adenoviruses (Ads) are important causes of respiratory tract infections and are responsible for 5 to 10% of all lower respiratory tract infections (LRTIs) occurring in infants and children (19). Fifty-one human adenovirus serotypes have, thus far, been identified (8) on the basis of their resistance to neutralization by antisera to other known Ad serotypes. These serotypes have been classified into six species, Ad A to F, on the basis of their biological and/or physicochemical characteristics.

Among the recognized serotypes, certain serotypes are frequently associated with severe disease. Severe acute respiratory illnesses have been associated with adenovirus type 1 (Ad1) through Ad8, Ad19, Ad21, Ad35, and others, but Ad7 and, to a lesser extent, Ad3, both of which belong to species B, are most frequently associated with severe disease. Although Ad3 and Ad7 infections typically result only in mild upper respiratory tract illnesses and conjunctivitis, these infections can also culminate in more serious LRTIs, disseminated disease, and even death, particularly in infants and persons with underlying immunological or pulmonary problems (7, 12, 36). Severe infections can occur in previously healthy children as well. Severe adenoviral infections can also occur as an outbreak or epidemic. Outbreaks of severe infections in healthy children are most frequently reported in association with Ad7, followed by Ad3 and Ad21 (15, 16, 18).

Since 1990, when the Seoul National University Children's Hospital initiated viral surveillance for childhood LRTIs, a large nationwide epidemic of Ad7-associated pneumonia was first reported from 1995 to 1997 (15), and more limited epidemics were noted every 1 to 2 years thereafter. On the other hand, Ad3 strains were isolated sporadically until 1995, and outbreaks of LRTIs associated with Ad3 have occurred every 1 to 2 years since 1998. Overall, these two serotypes accounted for more than half of the adenoviral LRTIs which were diagnosed at the institute.

The isolation of Ads, as well as the determination of serotypes via neutralization tests or hemagglutinin inhibition with type-specific antisera, is a process that may take several weeks (32). The rapid diagnosis of adenoviral respiratory infections, as well as the determination of serotypes, is crucial to epidemiological surveillance and to decisions regarding optimal treatment strategies, as specific serotypes are frequently associated with the manifestation and severity of the disease (30). This necessity has led us to develop rapid diagnostic assays for Ads. The shell vial method improves isolation in tissue cultures by increasing viral adsorption to the cells. However, it still requires an assay time of approximately 2 days for most serotypes (22) and does not allow discrimination among the Ad serotypes. Although the direct antigen detection assay, based on immunofluorescence or enzyme-linked immunosorbent assays, can also be used for the rapid diagnosis of Ad infections, they appear to be less sensitive than virus culture assays, and the results can vary considerably (28, 34). Serological tests normally require documentation of an increase in antibody titer from an acute-phase to a convalescent-phase blood sample; thus, test results tend to come in too late to be relevant to the treatment of acute disease (13).

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Recently, the PCR-based assay has become a popular alternative method for the detection and typing of Ads, offering rapid and sensitive detection and precise molecular identification. Many attempts have been made to detect Ads directly from clinical specimens (1, 5, 9, 11, 17, 27) and to identify species or selected serotypes by PCR-based assays of viral isolates or clinical specimens. Most of these attempts have required further characterization of the amplicons, including restriction enzyme analysis, hybridization with probes, sequencing (4, 5, 6, 11, 25, 30, 33), or additional type-specific PCR (26) for the identification of species or serotypes. Two studies used multiplex PCR assays in the identification of selected Ad serotypes on the basis of amplicon size without further investigation (21, 35). However, these strategies were applied only to viral stocks and were never tested on clinical specimens.

This study was performed, then, in order to develop a single-step multiplex PCR assay that is able to detect all Ad serotypes and to discriminate between Ad3 and Ad7 directly from clinical specimens. The reliability and accuracy of this single-tube multiplex PCR method were evaluated using both diverse viral isolates and respiratory specimens.

MATERIALS AND METHODS

Clinical specimens and viruses. (i) Clinical specimens. A database had previously been established for children suffering from LRTIs, who had been diagnosed at the Seoul National University Children's Hospital, Seoul, Korea, from 1990 to 2004 (15). This database included viral isolates from patients, clinical diagnoses, and demographic data. Nasal aspirate specimens were collected with mucus traps and catheters. The specimens were refrigerated immediately after collection and processed in the laboratory within 72 h. The specimens were divided into three portions. One portion was used for antigen detection via indirect immunofluorescent antibody staining, employing murine monoclonal antibodies (Chemicon, Temecula, CA) and fluorescein isothiocyanate-conjugated goat anti-murine immunoglobulin G (Cappel, West Chester, PA). The second portion was used for the isolation of major respiratory viruses via inoculation into monolayers of HEp-2, MDCK, and LLC-MK2 cells, and the remaining portion was kept frozen at −70°C for later use.

One hundred twenty-seven respiratory specimens from children suffering from LRTIs from which Ads had been isolated were included in this study. The original clinical specimens previously grew the following Ad serotypes (determined by neutralization tests [14, 15]): Ad1 (number of specimens [*n*] = 13), Ad2 (*n* = 19), Ad3 (*n* = 29), Ad4 (*n* = 8), Ad5 (*n* = 7), Ad6 (*n* = 4), Ad7 (*n* = 36), Ad11 (*n* = 3), and some other Ad serotypes (*n* = 8). For a negative control, 53 nasal aspirates of children with LRTIs from which respiratory agents other than Ad were isolated or for whom no etiologic diagnosis was made were also included in this study and designated as follows: specimens that yielded no specific agents (*n* = 20), influenza virus type A (*n* = 2) or B (*n* = 1), respiratory syncytial virus group A or B (*n* = 8), parainfluenza virus type 1, 2, or 3 (*n* = 3), *Streptococcus pneumoniae* (*n* = 1), and *Mycoplasma pneumoniae* (*n* = 18). The specimens were kept frozen for up to 10 years, and some of them were thawed and frozen several times before processing for this study.

(ii) Virus isolates. (a) Viral culture and identification. Nasal aspirate specimens were inoculated into HEp-2, MDCK, and LLC-MK2 cell culture tubes. Adenovirus isolates were identified on the basis of their characteristic cytopathic effects following the microscopic examination of cell monolayers every 2 to 3 days during a 4-week incubation at 37°C. Viral identification was confirmed by virus-specific indirect immunofluorescence staining. The isolates were kept frozen at −70°C.

(b) Serotyping of adenoviruses. Adenoviral serotypes were identified by a neutralization assay with type-specific reference antisera (14, 24). Antisera against types 1 to 7 and 11 were employed, and isolates which could not be neutralized by these eight antisera were designated unusual types.

A total of 106 adenoviral isolates, which were obtained from children with LRTIs, were included in this study. The identified serotypes were as follows: Ad1 (*n* = 4), Ad2 (*n* = 4), Ad3 (selected from different genome types of Ad3, Ad3a13 through Ad3a18; *n* = 28) (16), Ad4 (*n* = 16), Ad5 (*n* = 4), Ad6 (*n* = 6), Ad7 (genome types Ad7d and Ad7l; *n* = 12) (16), Ad11 (*n* = 8), and Ads of unusual types (*n* = 24). Some of the adenoviral isolates were matched to clinical specimens included in this multiplex PCR study and the others were not. Six strains of other respiratory viruses, influenza virus type A and B (*n* = 2) and respiratory syncytial virus (*n* = 4) grown in MDCK cells or HEp-2 cells, as well as two specimens from which *M. pneumoniae* were detected by PCR, were also included for assessment of the specificity of PCR results.

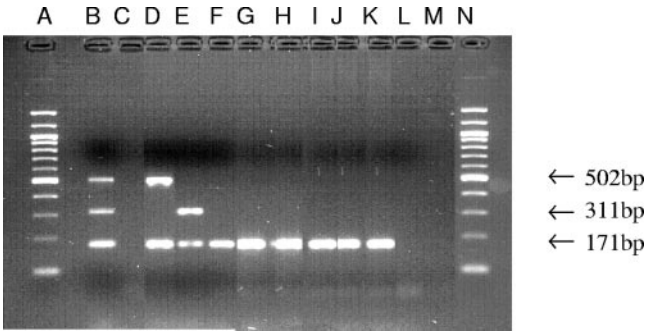

FIG. 1. Amplification by consensus primers and Ad3- and Ad7-specific primers in one-step multiplex PCR with clinical specimens. Lane A, 100-bp ladder; lane B, positive control (a mixture of Ad3 and Ad7 strains); lane C, negative control; lane D, Ad3; lane E, Ad7; lane F, Ad1; lane G, Ad2; lane H, Ad5; lane I, Ad6; lane J, Ad11; lane K, Ad of unusual type; lane L, respiratory syncytial virus; lane M, *M. pneumoniae*; lane N, 100-bp ladder. A total of 180 clinical specimens were tested in this study; 10 clinical specimens are shown here.

Multiplex PCR. (i) Preparation of DNA. Ad isolates which had been stored at −70°C were subsequently propagated on HEp-2 cell monolayers in a tube culture. Infected-cell scrapes were boiled at 95°C for 5 min and centrifuged at 1,300 rpm for 5 min. The supernatant containing viral DNA was used as a PCR template. Other respiratory virus strains, which were used for negative controls, were treated similarly to the adenoviral isolates, using appropriate cell lines.

The extraction and purification of DNA from the clinical specimens were performed as described in the QIAamp blood mini kit (QIAGEN GmbH, Hilden, Germany). Briefly, 20 μl of proteinase K was added to each 200-μl sample. Buffer AL was also added to the sample; the sample and buffer were subsequently mixed, incubated at 56°C for 10 min, and then centrifuged. Ethanol (100%) was added, the mixture was applied to the QIAamp spin column, and the column was washed with AW1 and AW2 buffers. Finally, viral DNA was eluted with AE buffer.

(ii) Primers. The primers used in this study were as follows. Consensus primers for the detection of all Ad serotypes were based on the hexon sequence, corresponding to a conserved region of the hexon gene upstream of surface loop 1, and amplifying all 51 Ad serotypes, as described by Allard et al. (4), with some minor modifications. The primer pair, nehex3deg (5′-GCCCGYGCMACNGAN ACSTACTTC-3′) and nehex4deg (5′-CCYACRGCCAGNGTRWANCGM RCYTTGTA-3′), produced a 171-bp amplimer. The sequences for the type-specific primers (Ad3F and Ad3R for Ad3; Ad7F and Ad7R for Ad7) were published by Xu and Erdman in 2001 (35), and the primers were designed on the basis of the hypervariable regions of hexon gene loop 1 and 2 of each type of Ad. Twenty-nine strains of Ad3 and 12 strains of Ad7, all of which were isolated in Korea (16), were sequenced, and no variations were found in the regions in which the primers were designed (E. H. Choi and H. J. Lee, unpublished data). The amplicon size of 502 bp indicated the generation of an Ad3-specific product, whereas an amplicon size of 311 bp indicated the generation of an Ad7-specific product (Fig. 1).

(iii) Multiplex PCR amplification. Single-step amplifications were carried out in 10-μl reaction mixtures. The 10-μl reaction mixtures contained the following ingredients: double-distilled H_2O, 3.8 μl; 10× Tris-HCl (pH 8.3) reaction buffer, 1.0 μl; 25 mM $MgCl_2$, 0.7 μl; deoxynucleoside triphosphate (10 mM), 0.2 μl; 5 U/μl AmpliTaq Gold (Applied Biosystems, Inc., Foster, CA), 0.1 μl; and three primer sets (Ad3F/Ad3R, Ad7F/Ad7R, and nehex3deg/nehex4deg), 0.2 μl, respectively. Three microliters of the supernatant of the boiled-cell scrapings (of clinical isolates) or DNA extract (of clinical specimens) was added to each reaction mixture. The reaction tubes were then placed in a PTC-200 Peltier Thermal Cycler DNA engine (MJ Research, Watertown, MA) and were maintained at 95°C for 10 min, immediately followed by 35 cycles. One cycle consisted

of 95°C for 45 seconds, 55°C for 45 seconds, and 72°C for 45 seconds. The final cycle had a prolonged extension time of 7 min.

Each PCR test included distilled water as a negative control and mixed Ad3 and Ad7 DNA extracted via a modified Hirt procedure (31) as a positive control. When any PCR product was not visualized, the quality of the DNA in each specimen was assessed with an internal control, a primer set for the mitochondrial cytochrome oxidase subunit 3 gene (Cyt3_F [5′-ATGACCCACCAATCA CATGCCTATCA-3′] and Cyt3_R [5′-ACTAGTTAATTGGAAGTTAACGGT ACTA-3′]). To avoid potential contamination during DNA amplification, we ensured DNA-free handling during the preparation of the reaction mixture. All plugged pipette tips, tubes, and other materials used for PCR were disposable.

(iv) Gel electrophoresis. After the completion of amplification, 6 μl of the PCR product was run on a 2% agarose gel, containing 0.5 μl of ethidium bromide per μl, in 0.5× Tris buffer (40 mM Tris-acetate buffer, 2 mM EDTA [pH 8.3]) at 100 V for 40 min. The bands were visualized and analyzed under UV light.

(v) PCR product sequencing. PCR products of direct clinical specimens which evidenced amplimers discrepant from the neutralization test results of the isolated Ad strains were sequenced via the dideoxy chain termination method. The PCR products were pretreated with exonuclease I and shrimp alkaline phosphatase enzymes. Three primer sets, which included both forward and reverse primers, were used for bidirectional sequencing.

TABLE 1. Results of amplification by consensus primer in the multiplex PCR assay, using three pairs of primers for adenoviruses (i.e., consensus primers and Ad3- and Ad7-specific primers) and original clinical specimens

Amplification by consensus primers	No. of specimens that previously grew an adenovirus[a]	No. of specimens that did not grow adenovirus[b]	Total no. of specimens
Positive	115	2[c]	117
Negative	12[d]	51	63
Total	127	53	180

[a] Included specimens that grew Ad1 ($n = 13$), Ad2 ($n = 19$), Ad3 ($n = 29$), Ad4 ($n = 8$), Ad5 ($n = 7$), Ad6 ($n = 4$), Ad7 ($n = 36$), Ad11 ($n = 3$), and Ads of unusual types ($n = 8$).

[b] Included specimens that yielded no specific agents ($n = 20$), influenza type A ($n = 2$) and B ($n = 1$), respiratory syncytial virus group A or B ($n = 8$), parainfluenza virus types 1, 2 and 3 ($n = 3$), *Streptococcus pneumoniae* ($n = 1$), and *Mycoplasma pneumoniae* ($n = 18$).

[c] Included one specimen that yielded influenza virus type A and one specimen with no specific agents identified, and amplicons of consensus primers from these specimens were confirmed by sequencing analysis to belong to Ad subgroup C.

[d] Included specimens that grew Ad1 ($n = 5$), Ad2 ($n = 1$), Ad3 ($n = 1$), Ad5 ($n = 3$), Ad7 ($n = 1$), and Ads of unusual types ($n = 1$).

RESULTS

Evaluation of the multiplex PCR method for viral isolates. As a preliminary study for the evaluation of the utility of this single-tube multiplex PCR method, using three primer sets for clinical specimens, initially the viral isolates which had been kept frozen at −70°C were tested. The primers used in this study were evaluated in multiplex combinations under a variety of amplification conditions in order to obtain optimal results with each representative Ad type. The final multiplex PCR assay, performed under optimized conditions (i.e., 1.75 mM Mg^{2+} concentration and an annealing temperature of 55°C), was tested with viral isolates obtained from Korean children.

All 106 adenoviral isolates were successfully amplified with consensus primers, and the Ad3 and Ad7 serotypes were correctly matched with their neutralization test results. None of the isolates evidenced discordant results with the neutralization test results. All of the other respiratory viruses or bacteria proved to be negative.

Application of single-step multiplex PCR to original clinical specimens. In order to evaluate the usefulness of our multiplex PCR method for the rapid detection and identification of Ad3 and Ad7 directly from nasal aspirate specimens, we performed the assay on 127 clinical specimens from which Ads had been isolated and 53 nasal aspirates from which other respiratory agents and none of the viruses had been identified.

Amplification by consensus primers in multiplex PCR. Among the 127 clinical specimens from which an adenovirus was grown, 115 specimens were amplified to produce 171-bp amplicons via multiplex PCR (Table 1). Thus, the sensitivity of the assay with regard to the detection of any Ad serotypes directly from clinical specimens in the single-step multiplex PCR was 91% (115/127). Eleven of the 12 specimens with negative PCR results on multiplex PCR were successfully amplified, when we employed different reaction conditions with consensus primers only (i.e., higher Mg^{2+} concentration and absence of Ad3- and Ad7-specific primers), but these conditions were not appropriate for multiplex PCR, due to overwhelming nonspecific reaction products. The serotypes of Ads previously grown from the 12 specimens were Ad1 ($n = 5$), Ad2 ($n = 1$), Ad3 ($n = 1$), Ad5 ($n = 3$), Ad7 ($n = 1$), or Ad of unusual types ($n = 1$). It is noteworthy that 63 of the 65 specimens (97%) that grew Ad3 or Ad7 produced consensus primer amplicons, whereas 52 of 62 specimens (84%) that grew Ad serotypes other than Ad3 or Ad7 produced 171-bp-sized consensus primer amplicons.

Among the 53 specimens which did not grow Ads, two specimens produced 171-bp amplicons. The amplicons were proved by sequencing analysis to be Ad species C. One of the specimens had previously been shown to grow influenza virus, and the other grew no virus.

Amplification by Ad3- and Ad7-specific primers in multiplex PCR. Among the 115 clinical specimens that produced consensus primer amplicons, 28 nasal aspirates that grew Ad3 and 35 nasal aspirates that grew Ad7 were included. Multiplex serotype-specific PCR successfully identified 26 Ad3 serotypes and 35 Ad7 serotypes from the original clinical specimens, representing 93% sensitivity (26/28) for Ad3 and 100% (35/35) sensitivity for Ad7 (Table 2). None of the 12 specimens that were not amplified by consensus primers produced any Ad3- or Ad7-specific primer amplicons as the result of multiplex PCR. Consequently, the assay had a sensitivity of 90% (26/29) for discrimination of Ad3 and a sensitivity of 97% (35/36) for discrimination of Ad7 from all the original clinical specimens included in this study.

The multiplex PCR results of five specimens that previously grew Ad exhibited discordant results from the neutralization test results of the isolated strains (Table 3). Two specimens (obtained from patients 1 and 4 in Table 3) that previously grew Ad2 and Ad5, respectively, produced a 311-bp amplimer, as well as a 171-bp amplimer. The virus isolates grown from these specimens produced only the 171-bp amplimer by multiplex PCR. However, one of the virus isolates (Ad5 via neutralization test) exhibited a 311-bp band, corresponding to Ad7, on optimized PCR with only one pair of Ad7-specific primers, while the other (Ad2) did not. Sequencing analysis revealed that the 311-bp amplimers of these specimens were Ad7 specific. Also, additional sequencing analysis of the PCR bands produced by hex1deg (5′-GCCSCARTGGKCWTACAT

TABLE 2. Discrimination of Ad3 or Ad7 from Ads of other types in one-step multiplex PCR of 115 direct clinical specimens that produced amplicons of consensus primers

Multiplex PCR result	No. of specimens that previously grew the following Ad type[a]:			Total no. of specimens
	Ad3 (n = 28)	Ad7 (n = 35)	Ads of other types (n = 52)	
Amplification by Ad3-specific primers	26	1[b]	0	27
Amplification by Ad7-specific primers	2[c]	35[b]	2[d]	39

[a] Ad type determined by neutralization test.
[b] One specimen amplified 502-bp, 311-bp, and 171-bp bands. These 502-bp and 311-bp amplimers produced by multiplex PCR of clinical specimens were confirmed by sequencing analysis to be specific to Ad3 and Ad7, respectively.
[c] These two specimens amplified only the Ad7 band (311-bp size) and the Ad common bands on multiplex PCR, and the 311-bp-sized products were compatible with Ad7 bands via sequencing analysis. However, both of the specimens exhibited Ad3 bands upon optimized PCR with Ad3F/Ad3R primers.
[d] Two specimens that previously grew Ad2 (n = 1) and Ad5 (n = 1) produced 311-bp amplimers, as well as 171-bp amplimers. The sequencing of 311-bp amplimers of these specimens revealed that the 311-bp amplimers were specific to Ad7. Also, additional sequencing analysis of the 301-bp bands produced by hex1deg and hex2deg by Allard et al. (4) revealed Ad2- and Ad5-specific products, respectively.

GCACATC-3′) and hex2deg (5′-CAGCACSCCNCGRATGTC AAA-3′), which were used by Allard et al. as outer primers for nested PCR (4) and yielded a 301-bp product, revealed Ad2-specific and Ad5-specific products, respectively.

Two specimens (obtained from patients 2 and 3) that had previously been shown to grow Ad3 produced 311-bp and 171-bp bands but did not produce 502-bp amplicons as the result of multiplex PCR, and the 311-bp-sized products were shown to be compatible with the Ad7 bands via sequencing analysis (patients 2 and 3). The viral isolates grown from these two specimens produced 502-bp and 171-bp amplimers, without any 311-bp band, via multiplex PCR. When optimized PCR with only one primer set (Ad3F/Ad3R or Ad7F/Ad7R) was performed, one (patient 3) of the viral isolates exhibited Ad3 and Ad7 bands, and the other (patient 2) showed only an Ad3 band. However, both of the nasal aspirate specimens evidenced Ad3 bands (502-bp amplicons) upon the performance of optimized PCR with Ad3F/Ad3R primers.

TABLE 3. Identification of mixed adenoviral infections of two different serotypes by multiplex PCR assay

Patient	Type(s) of viral isolates determined by:		Type(s) of Ad in the clinical specimens determined by:		Confirmed serotypes
	Neutralization test	Sequencing of PCR products[a]	Multiplex PCR[b]	Sequencing of PCR products	
1	2	2	7	2, 7	2, 7
2	3	3	7	3, 7	3, 7
3	3	3, 7	7	3, 7	3, 7
4	5	5, 7	7	5, 7	5, 7
5	7	7	3, 7	3, 7	3, 7

[a] The sequencing result was confirmed bidirectionally by the use of different PCR products which are common to adenovirus, type 3 and type 7 strains, as appropriate.
[b] Identical results were obtained in multiple experiments by repeating the DNA extraction procedure with the clinical specimens and different stocks of the viral isolates which were kept separately. Additionally, optimized PCR with Ad3F/Ad3R primers or Ad7F/Ad7R primers was performed repeatedly (see the text).

The last specimen (obtained from patient 5) that exhibited a discordant multiplex PCR result for the clinical specimen from the viral isolate neutralization test had previously grown Ad7, but the multiplex PCR assay resulted in the formation of three bands, 502-bp, 311-bp, and 171-bp bands. The viral isolates grown from this specimen produced only 311-bp and 171-bp amplicons upon multiplex PCR, and optimized PCR of the viral isolates with Ad3FR exhibited no Ad3 band. These 502-bp and 311-bp-sized amplimers, which were produced by the multiplex PCR of clinical specimens, were also confirmed via sequencing analysis to be specific for Ad3 and Ad7, respectively.

Thus, we concluded that the five specimens discussed above included more than one type of Ad, although culture and neutralization tests identified only one type of Ad in each specimen.

DISCUSSION

The identification of Ad serotypes usually relies on cell culture-based type-specific neutralization, which is in turn based on hemagglutinin inhibition or neutralization tests of the isolates. However, these assays are quite time-consuming and depend on a sufficient yield of infectious viruses and, consequently, are not suitable for the rapid and sensitive identification of Ads. The detection and identification of Ads in patients' specimens via PCR-based assays have become popular alternative methods that allow rapid, sensitive, and precise molecular identification. In many studies, PCR has been used for the detection of Ads directly from clinical samples of different origins (1, 5, 9, 11, 17, 20, 27). The sensitivity of PCR with regard to detection of Ads compared to the culture method ranged from 76% to 100%, depending on the specimens, primers, and/or reaction conditions, etc., employed. Recently, the introduction of real-time PCR increased the sensitivity and speed of Ad detection (11). Compared to the culture method, the Ad detection sensitivity achieved by the multiplex PCR method employed in this study was 91%. Although 11 of the 12 specimens with negative results in single-step multiplex PCR could be amplified using optimized PCR with consensus primers only, these reaction conditions could not be employed in the multiplex PCR due to the abundance of nonspecific amplifications.

PCR-based identification systems for Ad species have been developed recently and used in several studies. Species may be differentiated by restriction enzyme analysis of the amplicons produced by general PCR (3, 10) or by size or hybridization patterns with probes of the amplicons produced by multiplex PCR (23, 27). However, species-specific PCR is also associated with significant limitations. For example, this technique is unable to distinguish between the epidemic keratoconjunctivitis-causing adenoviruses and other species D adenoviruses, which cause a relatively harmless form of conjunctivitis.

The determination of a few selected serotypes can be accomplished by restriction enzyme analysis of the PCR product (4, 6, 29) or by additional type-specific PCR after the initial PCR for the presence of Ads using genus-specific primers (26). In order to identify diverse serotypes, a sequence analysis of

the amplicons, normally a selected hypervariable region of the hexon, is required (29, 30).

Ad3 and Ad7 have been responsible for severe epidemics of pneumonia in children. In Korea, severe LRTIs caused by these serotypes were associated with significant childhood morbidity, as well as relatively high mortality rates (15, 16). Furthermore, these two serotypes are predominantly isolated, accounting for over half (54%) of total Ad isolates from children suffering from LRTIs at the Seoul National University Children's Hospital, Seoul, Korea, from 1990 to 2004 (unpublished data). The establishment of a method for the rapid diagnosis of Ad3 and Ad7 infections is considered essential for the development of optimal treatment strategies for each patient, as well as for the evaluation of potentially beneficial therapeutic modalities.

There have been two studies of multiplex PCR assays in which the ability of the technique to identify selected Ad serotypes on the basis of amplicon size, without further characterization of the amplicons, was examined (21, 35). Xu and Erdman (35) designed a multiplex PCR method that could identify Ad3, Ad7, and Ad21. The multiplex PCR developed by Na et al. (21) was able to identify Ad1, Ad2, Ad3, Ad4, Ad5, and Ad7. However, both of these studies were primarily concerned with viral isolates. Multiplex PCR studies using original nasal aspirates obtained from young patients with LRTIs have not, until now, been attempted.

In this study, we designed a single-tube multiplex PCR assay for the detection of Ads and identified Ad3 and Ad7 without performing viral isolation and neutralizing tests, and we attempted to evaluate the usefulness of this multiplex PCR method on clinical specimens which had been obtained from children suffering from LRTIs.

As a preliminary study for the evaluation of the accuracy and reliability of this single-tube multiplex PCR, we tested 106 adenoviral isolates; the sensitivity and specificity with which our method could identify Ads and discriminate between Ad3 and Ad7 were both 100%.

In this study, multiplex PCR on nasal aspirate specimens evidenced relatively good reliability; the sensitivity of this technique in the detection of Ads directly from clinical specimens was 91%. Two apparently false-positive specimens were confirmed to contain Ads via amplicon sequencing analysis. Therefore, the specificity of this method was found to be 100%. The method's sensitivities with regard to the identification of Ad3 and Ad7 among specimens that produced consensus primer amplimers was 93% for Ad3 and 100% for Ad7. It is notable that a relatively higher percentage of specimens that grew Ad types other than Ad3 or Ad7 compared to the specimens that grew Ad3 or Ad7 exhibited negative PCR results with consensus primers and multiplex PCR (16% versus 3%). This may be attributable, in part, to one of the biological characteristics of Ads, namely, that many Ad types, particularly the common childhood types (Ad1, Ad2, and Ad5) (2), are often associated with shedding for a prolonged period of time (19) and, thereby, may be present at low titers in nasal secretions.

The multiplex PCR assay developed in this study may not be ideal for detection of Ads of all types, considering the sensitivity of consensus primers (91%). However, there are differences in the level of reduced sensitivity among the serotypes. As mentioned above, the sensitivity for detecting Ads other than Ad3 or Ad7 decreased to 84% (52/62). In contrast, the sensitivity for detecting Ad3 or Ad7 by using consensus primers still remained as high as 97% (63/65) in the multiplex PCR, and the sensitivity for discriminating between Ad3 and Ad7 by type-specific primers was 94% (61/65; 90% [26/29] for Ad3 and 97% [35/36] for Ad7) for all original clinical specimens. In particular, this study was undertaken to develop a rapid assay to identify Ad3 or Ad7 infections, which often cause severe infections and may require prompt decisions regarding treatment strategies. Although we observed a slight decrease in the sensitivity in detecting Ads overall, this multiplex assay is convenient and useful with a high sensitivity for Ad3 and Ad7 at least.

The multiplex PCR assay with the original clinical specimens indicated that five out of 115 specimens contained more than one type of Ad, although the culture and neutralization tests identified only one type in the specimens. Wu et al. reported infections with multiple Ad serotypes in an abstract (J. Wu, N. Freed, C. Le, M. Ryan, A. Hawksworth, and K. Russell, Abstr. 41th Annu. Meet. Infect. Dis. Soc. Am., abstr. 342, 2003), but detailed information is not yet available. It is conceivable that some results which suggest infections with multiple Ad types may be due to contamination of the original specimens or viral isolates with other types of Ad, possibly occurring during handling over the 10 years spent in storage or during the PCR assay procedure. In order to minimize this possibility, all of our procedures were confirmed by repeating the experiments from the DNA extraction steps for the clinical specimens and repeating them with different stocks of the same strain for the viral isolates. The systems used in this study were able to detect only Ad3 and Ad7, and our assay was able to identify mixed infections only with regard to Ad3, Ad7, and a select group of other types. Therefore, coinfections with serotypes other than Ad3 and Ad7, including Ad2, Ad5, etc., could not be discerned by the use of our assay method. The level to which coinfection by different serotypes was detected in this study may represent an underestimate of the true total, and a host of further assays will be required before the total amount of coinfections can be elucidated.

In conclusion, the single-step multiplex PCR developed in this study, which involved one consensus primer set and two type-specific primer pairs, proved able to enhance the rapidity of Ad detection, as well as the determination of Ad3 or Ad7 directly from clinical specimens. Lastly, this method may be applied to future investigation, potentially facilitating the improvement of epidemiological investigations, and the development of potentially beneficial therapeutic strategies for Ad3 and Ad7 infections, all of which can cause severe disease.

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