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Research Article

Identification and Typing of Respiratory Adenoviruses in Guangzhou, Southern China Using a Rapid and Simple Method*

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Human adenoviruses (HAdVs), especially HAdV-B3, -E4 and -B7, are associated with Acute Respiratory Disease in Chinese children, and occasionally in adults. In order to establish and document the profiles of the respiratory adenovirus pathogen among children in Guangzhou, Southern China, a rapid, simple and practical method for identification and typing of respiratory adenoviruses was developed and evaluated. One pair of universal PCR primers was designed according to the conserved region of the hexon gene, which can detect not only HAdV-B3, -E4 and -B7, but also HAdV-B14, -F40 and -F41, with a specific 300bp PCR product. Three pairs of type-specific PCR primers were also designed according to the hypervariable regions of the hexon gene to type HAdV-B3, -E4 and -B7 by three independent PCR reactions, making it easy to optimize the PCR conditions. By using this method, one hundred throat swab specimens collected during Oct 2010 to Dec 2011 and suspected of being positive for adenoviral infection were identified and typed for adenoviruses. Of these samples, fifty-five were adenovirus-positive. The most common HAdV type was HAdV-B3, identified in 92.7% of samples, which is not only consistent with the data reported in 2004-2006, but also consistent with the recent report in Hangzhou, eastern China, indicating that HAdV-B3 has been circulating in Guangzhou, and maybe in eastern China, for many years. The method for the respiratory adenovirus identification and typing we developed is rapid, simple and practical, which has a potential in the real-time surveillance of circulating adenovirus strains and also to provide etiological evidence for the adenovirus-relative disease control and prevention in China.

Adenovirus; Molecular epidemiology; Identification; Typing; Southern China

Human adenoviruses (HAdVs) were initially characterized as respiratory tract pathogens in the early 1950s (Hllleman M R, et al., 1954; Rowe W P, et al., 1953). HAdV infections are responsible for a large spectrum of diseases in human, such as Acute Respiratory Disease (ARD), pharyngoconjunctivitis, acute hemorrhagic cystitis and infant gastroenteritis, with approximately 5-7% of respiratory illnesses in young children attributed to HAdVs (Carballal G, et al., 2001). Although most of respiratory infections caused by HAdVs are self-limiting, fatal infections also occur in children and adults (Carr M J, et al., 2011; Girouard G, et al., 2011; Rebelo-de-Andrade H, et al., 2010).

To date, 68 genotypes of HAdVs have been identified (GenBank accession no. JN860678) (Dehghan S, et al., 2012) and classified into 7 species: species A to G (Jones M S, II, et al., 2007). Human adenovirus genotypes 3, 4 and (HAdV-B3, -E4, and -B7) are the major HAdVs associated with ARD in children and adults and have caused many outbreaks of pneumonia and pharyngoconjunctivitis in

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China (Guo L, et al., 2012; Ou Z Y, et al., 2008; Tang L-y, et al., 2006; Tang L, et al., 2011; Zhang Q, et al., 2006). Therefore, it is of critical importance for clinic and disease prevention and control to identify and type HAdVs for the potential outbreaks.

Here, we developed a simple, rapid and practical detection and typing method for the surveillance of HAdVs in Guangzhou, Southern China. As the main ingredient of HAdV capsids, the hexon protein contains most of the type-specific epitopes that can stimulate the host to produce type-specific neutralizing antibodies. The differences amongst different types of HAdVs rely mainly on the hypervariable regions (HVRs) in the hexon gene. Based on the conserved region and HVRs, we designed one pair of universal primers and three pairs of type-specific primers, which can detect and type HAdV-B3, -E4 and -B7, predominant in China by traditional PCR. By using this method, the molecular epidemiology of HAdVs circulating in Guangzhou, Southern China during Oct 2010 to Dec 2011was performed.

MATERIALS AND METHODS

Viruses, specimens and other materials

The adenovirus strains of HAdV-B3, -E4, -B7 and -B14 were isolated, identified and preserved in our laboratory (Su X, et al., 2011; Zhang Q-W, et al., 2012; Zhang Q, et al., 2012; Zhang Q, et al., 2006). HAdV -F40 and -F41 were gifts that were isolated and identified by Professor Chen in Southern Medical University. Human rhinovirus and coxsackie virus type A16 were identified by Guangdong CDC. Human influenza A/H1N1, chlamydia and mycoplasma were identified by Guangzhou Women and Children's Hospital. Human respiratory syncytial virus strain Long was bought from the ATCC (Cat no. VR-26). One hundred throat swab specimens suspected to be associated with adenoviral infection were collected from the outpatient clinic and hospitalized children with bronchial pneumonia in Guangzhou Women and Children's Medical Center during Oct 2010 to Dec 2011, of which, 60 were male and 40 were female, and aged one month to eight years old. Taq Master Mix PCR kits and Viral DNA extraction kits were purchased from Omega Bio-Tek Inc. Corp (Norcross). DL1000 and DL2000 DNA Markers were the product of TAKARA Corp (Dalian).

Adenovirus Culture

Throat swabs were inoculated into A549 cells and cultured at 37 °C in an atmosphere containing 5% (v/v) carbon dioxide in Dulbecco's minimum essential medium supplemented with 100 IU penicillin per mL, 100 mg streptomycin per mL and 2% (v/v) fetal calf serum. The cells were observed for 1-2 weeks for CPE and identified by PCR or sequencing.

Viral DNA extraction and Primers

Viral nucleic acids from the throat swabs and adenovirus strains were extracted according to the E.Z.N.A. Viral DNA kit instructions (Omega Bio-tek, Inc.). Universal primers HexF and HexR were designed according to the conserved region of the hexon gene from all HAdV types, generating a 300 bp PCR product; Type-specific primers (Ad3F, Ad3R; Ad4F, Ad4R; Ad7F, Ad7R) amplifying the HVRs of the hexon gene were designed and optimized to correctly identify HAdV-B3, -E4 and -B7, respectively. The size of all the PCR products was about 300bp. All the primes used for detection and typing of HAdVs are shown in Table 1. The PCR reaction system was as follows: 2 × Taq Master Mix (10 μL), primer F (10 μmol/L, 0.5 μL), primer R (10

Table 1. Primes used for the detection, typing and sequencing of HAdVs

Primer names	Primer sequences	Positions in Genome	GenBank No.
HexF (Allard A, et al., 1990)	5'-GCCCCAATGGGCATACATGCACATC -3'	18442-18466	DQ099432
HexR	5'-AGCACGCCGCGAATGTCAAAG-3'	18741-18721	DQ099432
Ad3F	5'-AAGACATTACCACTACTGAAGGAGAAGAA-3'	18933-18961	DQ099432
Ad3R	5'- CGCTAAAGCTCCTGCAACAGCAT-3'	19246-19224	DQ099432
Ad4F	5'- AGCAAAATGCATACCTTTGGGG-3'	18665-18686	AY594253
Ad4R	5'- ATAGTTAGGAGTGGTGGCGGCG-3'	18988-18967	AY594253
Ad7F (Xu W, et al., 2001)	5'- GGGAAAGACATTACTGCAGACA-3'	18890-18911	AY594256
Ad7R	5'-AAAAAGCGTCAGCAGCTTCT-3'	19190-19171	AY594256
HVRF	5'-CAGGATGCTTCGGAGTACCTGAG-3'	18473-18495	DQ099432
HVRR	5'-TTTCTGAAGTTCCACTCGTAGGTGTA-3'	20157-20132	DQ099432

Primer HexF was optimized based on the primer described elsewhere (Allard A, et al., 1990). Primer Ad7F was optimized based on the primer of Xu and Erdman (Xu W, et al., 2001).

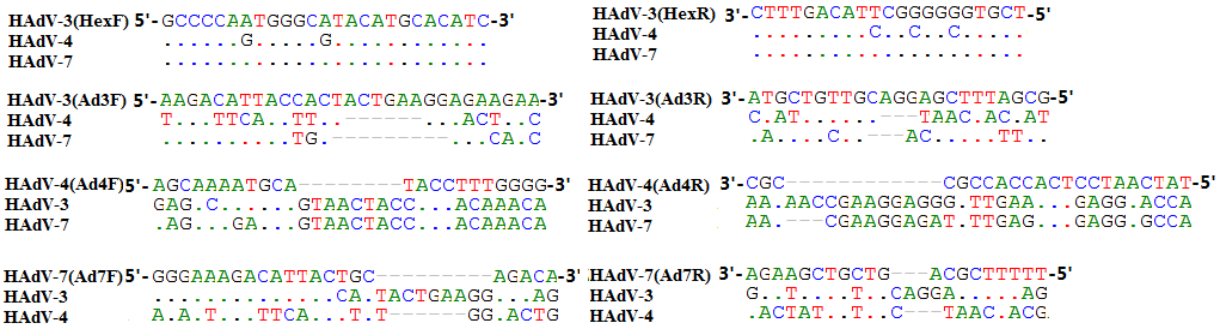

Fig. 1. Alignment of universal and type-specific primers of HAdV-3, -4 and -7. Two or three nucleotide differences at the 5 'end of primers HexF and HexR; three to nine nucleotide deletions or 2-4 nucleotide differences at the 3' end of primers Ad3F and Ad3R, Ad4F and Ad4R, Ad7F and Ad7R. HexF and HexR: universal primers; Ad3F and Ad3R: type-specific primers for HAdV-B3; Ad4F and Ad4R: type-specific primers for HAdV-E4; Ad7F and Ad7R: type-specific primers for HAdV-B7.

mmol/L, 0.5 μL), DNA template (2 μL) and sterile water (7 μL). PCR Reaction conditions for HAdV detection and typing is as follows: 94 ºC for 1 min, 34 cycles of 94 ºC for 30 s, 55 ºC for 30 s and 72 ºC for 20 s with a final extension of 72 ºC for 7 min. Primers HVRF and HVRR were designed according to the conserved region of the hexon gene and used for PCR and the sequencing of all seven HVRs of 1685 bp (Table 1). PCR was performed as follows: 94 ºC for 1 min, 34 cycles of 94 ºC for 30 s, 55 ºC for 30 s and 72 ºC for 100 s with a final extension of 72 ºC for 7 min.

Adenovirus detection and typing of clinical specimens

One hundred throat swab specimens suspected of being associated with adenoviral infection, collected during Oct 2010 to Dec 2011, were detected for presence of adenovirus and typed using our described method. PCR-Positive specimens were further cultured for 1-2 weeks in A549 cells for CPE observation.

Amplification and sequencing of HVRs of HAdV isolates

The HVRs of seven adenovirus isolates typed by type-specific primers were PCR amplified, purified and directly sequenced, including three of HAdV-B3, one of HAdV-E4, two of HAdV-B7 and one of HAdV-B14 selected randomly. The sequencing reaction was carried out by using an ABI Prism BigDye Terminator v3.1 Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase on an ABI 3730 DNA sequencer (Applied Biosystems). All of the sequences are sequenced at least three times.

RESULTS

Primer design

The universal amplification primers HexF and HexR of HAdV-B3, -E4 and -B7 were designed based on the conserved region of adenovirus hexon sequence (Fig. 1). Primer HexF was optimized based on the primer sequence described elsewhere (Allard A, et al., 1990). Primer Ad7F was optimized based on the primer of Xu and Erdman (Xu W, et al., 2001). According to the alignment of primer sequences, primers HexF and HexR are highly conserved in the majority of hexon genes, with only 2-3 nucleotide differences at the 5 'end, which ensures the effective PCR amplification of HAdV-B3, -E4 and -B7; the 3' terminal nucleotide sequences of type-specific primers vary significantly, with 3-9 nucleotide deletions or 2-4 nucleotide differences, which, in theory, ensures the DNA of one type of adenovirus can be PCR-amplified only by its type-specific primers and not by the other primers.

HAdV types 3, 4, 7, 14, 40 and 41 can be PCR-detected by universal adenovirus primers

The nucleus acids of two viral strains of HAdV-B3, -E4, -B7 and one viral strain of HAdV-B14, -F40 and -F41, were extracted and PCR-detected using universal primers HexF and HexR. All the adenovirus strains could

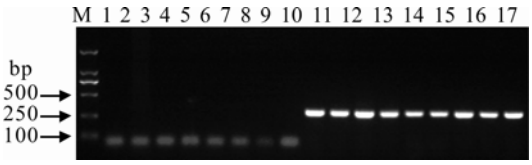

Fig. 2. Gel electrophoresis result of PCR amplification of nucleotide acids extracted from HAdV-B3, -E4, -B7, -B14, -F40, -F41 and other respiratory pathogens by universal primers HexF and HexR. The size of PCR bands is 300bp. M: DL2000 DNA Marker. 1: No-template control. 2: Mock-infected A549 cells. 3: Human influenza A/H1N1. 4: Human rhinovirus. 5: Human coxsackie virus CA16. 6: Human respiratory syncytial virus strain Long. 7: Chlamydia. 8: Mycoplasma. 9 and 10: HAdV-B3. 11 and 12: HAdV-E4. 13 and 14: HAdV-B7. 15: HAdV-B14. 16: HAdV-F40. 17: HAdV-F41.

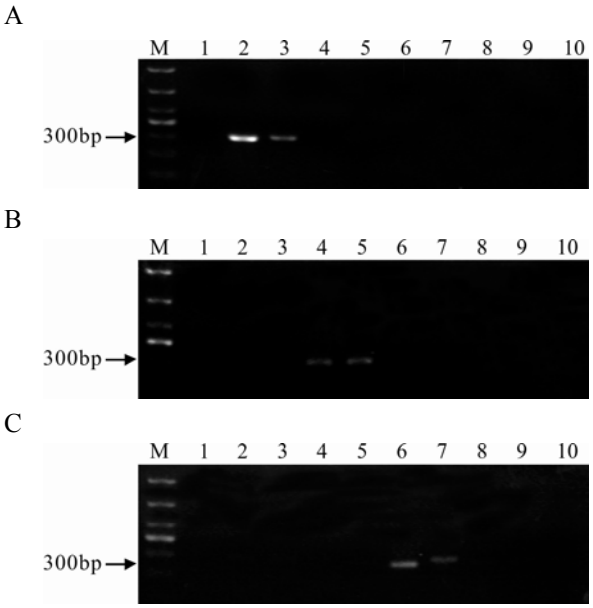

Fig. 3. Gel electrophoresis result of PCR amplification of nucleotide acids extracted from HAdV-B3, -E4, -B7, -B14, -F40 and -F41 by type-specific primers. A: PCR amplification result using primers Ad3F and Ad3R. B: PCR amplification result using primers Ad4F and Ad4R. C: PCR amplification result using primers Ad7F and Ad7R. M, DL1000 DNA Marker. 1, No-template control; 2 and 3, HAdV-B3; 4 and 5, HAdV-E4; 6 and 7, HAdV-B7; 8, HAdV-B14; 9, HAdV-E40; 10, HAdV-E41.

be detected by PCR amplification with specific 300 bp PCR bands (Fig. 2). No PCR bands were produced from other respiratory pathogen controls, indicating that this PCR method is specific. The result showed that HAdV types 3, 4, 7, 14, 40 and 41 can be PCR-detected specifically by our universal adenovirus primers.

HAdV types 3, 4 and 7 can be PCR-typed by type-specific primers

Two adenovirus strains of HAdV-B3, -E4, -B7 and one strain of HAdV-B14, -F40 and -F41 were PCR-typed by type-specific primers Ad3F and Ad3R, Ad4F and Ad4R, Ad7F and Ad7R, respectively. By using primers Ad3F and Ad3R, only HAdV-B3 could produce one 314bp PCR band; no PCR product was produced from HAdV-E4, -B7, -B14, -F40 and -F41 (Fig. 3A). For primers Ad4F and Ad4R, only HAdV-E4 strains could be PCR-identified with a 324bp band (Fig. 3B). Similarly, primers Ad7F and Ad7R were effective only in PCR-amplification of HAdV-B7 strains, with a PCR band of 301bp (Fig. 3C).

Molecular epidemiology of HAdVs circulating in Guangzhou, Southern China

One hundred throat swab specimens suspected of being associated with adenoviral infection were detected and

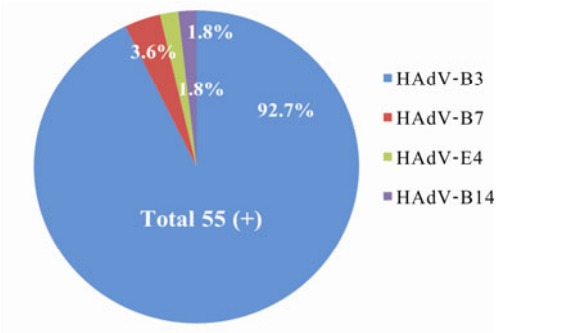

Fig. 4. HAdV types circulating in Guangzhou, Southern China during Oct 2010 to Dec 2011. HAdVs were detected and typed using universal and type-specific PCR. The percentage of each type was calculated relative to the number of total adenovirus-positive samples (55 samples).

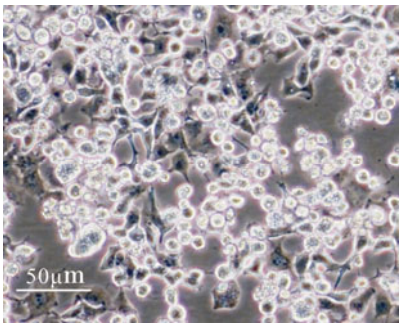

Fig. 5. CPE observed in A549 cells 96 h post-infection with one adenovirus PCR-positive throat swab specimen, which was identified as HAdV-B3 by type-specific primers (Scale Bar=50 μm).

typed for adenovirus by PCR using the method described above. Fifty-five of the 100 specimens were adenovirus-positive, of which, the most common (92.7%) HAdV type was HAdV-B3 (Fig. 4). Another two samples were identified as HAdV-B7 (3.6%), and the other was typed as HAdV-E4 (1.8%). One specimen which was adenovirus-positive could not be typed by our type-specific PCR primers, but identified as HAdV-B14 identified by subsequent full genome sequencing (Zhang Q, et al., 2012). Overall, species B was the most common HAdV species among children in Guangzhou, Southern China (98.2%). Among the 55 PCR-positive specimens, CPE was observed in 44 specimens by cell culture (isolation rate: 80%). Fig. 5 showed the typical CPE in A549 cells infected with one specimen which was identified as HAdV-B3. The subsequent sequencing and BLAST (blastn) analysis of HVRs of seven clinical isolates were consistent with the PCR-typing results, which further confirmed the reliability of our typing method.

DISCUSSION

HAdV types 3, 4 and 7 can cause serious clinical

symptoms, and occasionally with fatal consequences. However, the clinical symptoms of adenovirus infections are difficult to distinguish from those caused by other respiratory viruses or bacterial infection, which can result in delayed diagnosis and treatment. Currently, the clinical laboratory diagnoses of adenovirus infections rely mainly on cell culture or the ELISA method. However, cell culture is a time-consuming process and strict sterile conditions limit the use to clinical settings. Furthermore, the ELISA method is influenced by so many factors that it is prone to false-positive and negatives. Molecular biology techniques, especially PCR, which is sensitive and specific, have been widely used for the detection of HAdV infections.

In this study, by the alignment of hexon gene sequences of different HAdV types, universal primers were designed according to the conserved region, whereas type-specific primers were designed based on the HVRs. Six types of adenovirus can be detected by PCR with these universal primers, producing a specific PCR band, while no PCR bands were produced from other respiratory pathogens; The specific PCR bands were produced only by corresponding type-specific primers, indicating that these are of high specificity. The sensitivity of our method to adenovirus identification is higher than cell culture. Among 55 PCR-positive samples, only 44 were culture-positive.

The viral neutralization test is the traditional adenovirus typing method, which relies on the reaction between type-specific antibodies and type-specific antigenic determinants in hexon proteins (Madisch I, et al., 2005). However, it is hard to be applied in a clinical setting because of complicated operation procedures and lack of type-specific antibodies. Hexon gene sequencing and homologous analysis can also ascertain HAdV types (Biere B, et al., 2010). However, it costs too much and is time-consuming and so it does not meet the realities and needs for fast identification of the numerous clinical specimens. The nested PCR method developed in recent years has the advantages of fast, simple and specific characteristics, but it requires two rounds of PCR amplification with high risk of contamination (Deng J, et al., 2007). Moreover, since more than one pair of primers is used in one PCR reaction, competitions between them make it difficult to obtain the optimal conditions. Real-time fluorescent PCR is of high specificity, but the expensive instrumentation and probes hamper their common use in laboratories (Zhang Q W, et al., 2005). By contrast, the method we developed for identification and typing of respiratory adenoviruses has many advantages. Firstly, there is only one pair of primers in one PCR reaction, which makes it easy to optimize the PCR conditions. Secondly, three reactions can be performed in one PCR instrument simultaneously, saving at least half of the detection time compared to nested PCR and avoiding the risk contamination in the second nested PCR reaction. Thirdly, conventional PCR can be more accessible than real-time fluorogenic PCR especially in the standard laboratories. For the strains that cannot be typed by this method, sequencing of the hexon gene can be adopted for the final identification of adenovirus types, using the primers and PCR condition we supplied.

One hundred clinical throat swab specimens were detected and identified using this method. The data showed that HAdV-B, especially HAdV-B3 is the most common type circulating in Guangzhou, Southern China during Oct 2010 and Dec 2011, which is not only consistent with the results reported in 2004-2006 (Tang L-y, et al., 2006; Zhang Q, et al., 2006), but also consistent with the recent report in Hangzhou, eastern China (Xie L, et al., 2012), indicating that HAdV-B3 has been circulating in Guangzhou, Southern China, and may also have been present in eastern China for several years.

Our developed method can be applied for the rapid detection of adenovirus in the common laboratory conditions without the need for a fluorogenic quantitative PCR instrument, and also for molecular epidemiology or retrospective studies on a large number of adenovirus specimens. Furthermore, it is practical for the real-time surveillance of currently circulating adenovirus strains and the prediction of possible outbreaks of HAdV-related diseases. This in turn, will provide the etiological evidence for disease prevention and control.

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