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Discrimination between different types of human adeno-associated viruses in clinical samples by PCR

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Abstract

Persistent infection of human tissues with the helper virus-dependent parvovirus, adeno-associated virus (AAV) was detected by polymerase chain reaction (PCR) using primer pairs detecting AAV types 2, 3 or 5. In order to develop PCR protocols which discriminate between the different serotypes of AAV, the DNA of AAV-5 was sequenced partially and compared with the published sequences of AAV-2 and -3. Type specific oligonucleotides and specific probes which allow the distinction between human AAV types by PCR are described.

Keywords: Adeno-associated virus; AAV serotypes; PCR; Virus detection

1. Introduction

The human helper virus-dependent parvoviruses, adeno-associated viruses (AAV) have become increasingly interesting since they seem to be useful as gene transduction vectors targeting specific integration sites (for a recent review, see Flotte and Carter, 1995). AAV requires co-infection with helper viruses for replication. These can be adenovirus or viruses from the herpes family (reviewed in Berns and Bohensky, 1987), but also vaccinia virus (Schlehofer et al., 1986) and human papillomavirus (Walz et al., 1997) have been iden-

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Fig. 1. Sequence analysis of the AAV-5 *Bam*HI B fragment of AAV-5: The AAV-5 DNA *Bam*HI B fragment (Schlehofer et al., 1983; Bantel-Schaal and zur Hausen, 1984) was sequenced and aligned to the published sequence of AAV-2. The sequence stretch of 387 bp with less than 40% homology is shown in bold capitals. The locations of the primers, A5, pan1 and pan3 (Table 1; Fig. 3) are underlined. The type specific probes described in the text are doubly underlined.

tified as helpers. AAV infects humans early in childhood without known pathological consequences (Siegl et al., 1985; Murphy et al., 1995). Furthermore, AAV exhibits tumour suppressive properties (Schlehofer, 1994).

In recent years, PCR analyses have identified AAV sequences in human tissues including blood cells, biopsies and brushings from the *cervix uteri*, the endometrium and material from spontaneous abortion (Grossman et al., 1992; Tobiasch et al., 1994; Rabreau et al., 1995; Han et al., 1996; Friedman-Einat et al., 1997; Malhomme et al., 1997; Walz et al., 1997). Preliminary data suggest that, in addition, other organs are infected with AAV and may represent additional targets for natural AAV infection and/or latency (P. Klein-Bauernschmitt; O. Malhomme; (unpublished results)). However, the primers used in the cited studies did not discriminate between the three different types of human AAVs (types 2, 3, 5). In earlier studies, the different types of AAV have been distinguished by immunological methods detecting capsid protein variation (Atchison et al., 1965; Hoggan et al., 1966; Parks et al., 1967, 1970; Georg-Fries et al., 1984). AAV type 5 additionally demonstrates only weak cross-hybridisation with the other types (Bantel-Schaal and zur Hausen, 1984). Isolates of AAV types 2, 3 and 5 have been obtained from human clinical specimens, whereas types 1 and 4 appear to be of simian origin (Siegl et al., 1985; Murphy et al., 1995).

The complete DNA sequences of AAV-2 and -3 have been described (Srivastava et al., 1983; Cassinotti et al., 1988; Ruffing et al., 1994; Muramatsu et al., 1996). To date, information on the nucleotide sequence of the AAV-5 genome is not available. Restriction fragments of AAV-5 DNA showing a limited homology to AAV-2 are published (Bantel-Schaal and zur Hausen, 1984).

Using published nucleotide sequences for AAV types 2 and 3, and by cloning and sequencing the *Bam*HI B fragment of the genome of AAV type 5 (Schlehofer et al., 1983), we identified stretches of nucleotides different for the three virus types. This allowed us to construct primer pairs which are able to discriminate between the three virus types by PCR analyses of AAV infections in human biopsy material.

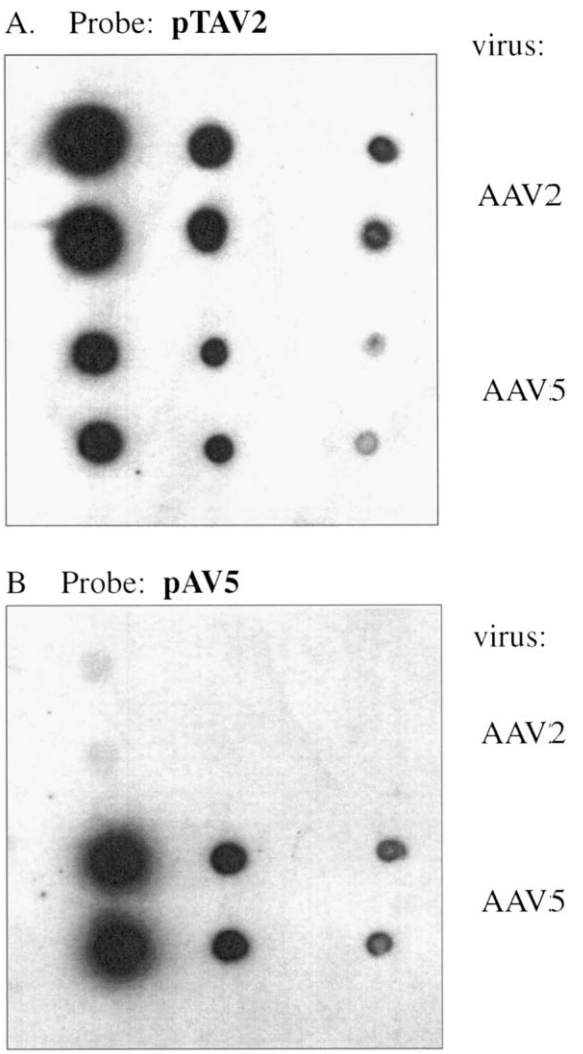

Fig. 2. Cross-hybridisation between AAV types: AAV-2 and -5 virions, respectively were purified (CsCl gradient centrifugation) from virus-infected HeLa cells (m.o.i.: 10 p.f.u./cell) after superinfection with adenovirus type 2 (m.o.i.: 1 p.f.u./cell). Purified virus (5 ml, corresponding to 1.5×10^6 (AAV-2) and 5×10^5 (AAV-5) infectious virions) was applied in 1:10 dilution steps to Nylon membranes (Gene-Screen, Dupont-NEN, Dreieich, Germany), denatured with 0.5 M NaOH, 1.5 M NaCl, neutralised with 0.5 M Tris–HCl (pH 7.3), 3 M NaCl and fixed by UV-crosslinking. The membrane was then saturated with 2XSSC, and prehybridised at 68°C for 2 h in 0.5 M Na_2HPO_4, 1 mM EDTA, 7% SDS. Hybridisation was performed overnight in the same solution, using radio labelled pTAV2 (containing the complete AAV-2 genome, Heilbronn et al. (1990) or pAV5).

Table 1
Nucleotide sequence and alignment of the primers, pan1, pan3 and b19 to the DNA sequences of AAV types and B19, respectively

		localisation	region	mismatches
Pan1	5` AACTGGACCAATTGAAAACTTTCC 3`			
AAV-2	----------G----------	1392-1415	p19-p40	1
AAV-3	----------	1389-1411	p19-p40	0
AAV-5	----------	?	p19-p40	0
B19	----------AT----------	1489-1511		2
Pan3	5` AAAAAGTCTTTGACTTCCTGCTT 3`			
AAV-2	----------	1706-1729	p19-p40	0
AAV-3	----------C----T----------T---	1703-1727	p19-p40	3
AAV-5	----------C----------	?	p19-p40	1
b19	5` TCCAGGCACAGCTACACTTCCAC 3`			
B19	----------	1644-1656		0

p19, p40 promotors (p) at map position 19 and 40

2. Partial sequence of AAV type 5

Using the T7 sequencing kit (Pharmacia, Freiburg, Germany) we determined the nucleotide sequence (Fig. 1) of the previously cloned *Bam*HI B fragment of AAV-5 (Schlehofer et al., 1983) which shows a relatively weak cross-hybridisation to AAV-2 DNA under stringent conditions (Bantel-Schaal and zur Hausen, 1984), and aligned the sequence to the published sequence of AAV-2. A stretch of 387 bp with less than 40% homology to AAV-2 DNA sequences (nucleotide position 1744–2152; including some alignment gaps) was identified. This 387 bp fragment was amplified by PCR and subcloned into the *Sma*I site of pUC18 (Messing, 1983) resulting in plasmid pAV5. Using pAV5 as a probe in Southern blot and slot blot analyses of AAV-2-infected cells, no hybridisation with AAV-2 fragments could be detected whereas the pTAV2 (Heilbronn et al., 1990) probe hybridised to both virus types, as expected (Fig. 2). Similarly, AAV-3 DNA did not cross-hybridise with pAV5 (data not shown).

3. Primer pairs discriminating between different AAV types

Sequences of high homology to AAV-2 within the *Bam*HI B fragment of AAV-5 could be identified which allowed the identification of general primer sequences detecting AAV-2, -3 and -5 by PCR analyses (Table 1). A pair of primers was determined comprising the nearly identical DNA sequences of the three virus types (pan1 and pan3; Tobiasch et al., 1994) amplifying AAV DNA of AAV-2, -3 or -5 in infected cells as well as in tissue biopsies. Pan1 is, in addition, homologous to sequences of the parvovirus B-19 (Shade et al., 1986) allowing detection of B-19 when using a B-19 specific right hand primer (b19; Table 1). Infections with B-19 are known to be involved in foetal disease and abortion (Brown et al., 1984; Anand et al., 1987), and AAV DNA was detected in material from spontaneous abortion (Tobiasch et al., 1994). The use of the three primers, pan1, pan3 and b19 allows to detect simultaneously the presence of AAV or B-19 DNA within the same PCR reaction since amplified sequences are distinct in size for AAV (338 bp) and B-19 (167 bp). AAV-2 can be distinguished from AAV-5 by hybridisation using an oligonucleotide located within the amplified fragment (AAV-2 specific oligonucleotide (position 1669–1688): 5′CACCCGCCGTCTGGATCATG3′; AAV-5 specific oligonucleotide (position 414–433): GACTAAGCGGCTCCCGGCCAG3′).

Using sequence information from the *Bam*HI B fragment of AAV-5, an AAV-5 specific sequence was identified allowing the construction of a primer (position 1267–1288, analogous to AAV-2 nucleotide positions, Fig. 3) which can be used

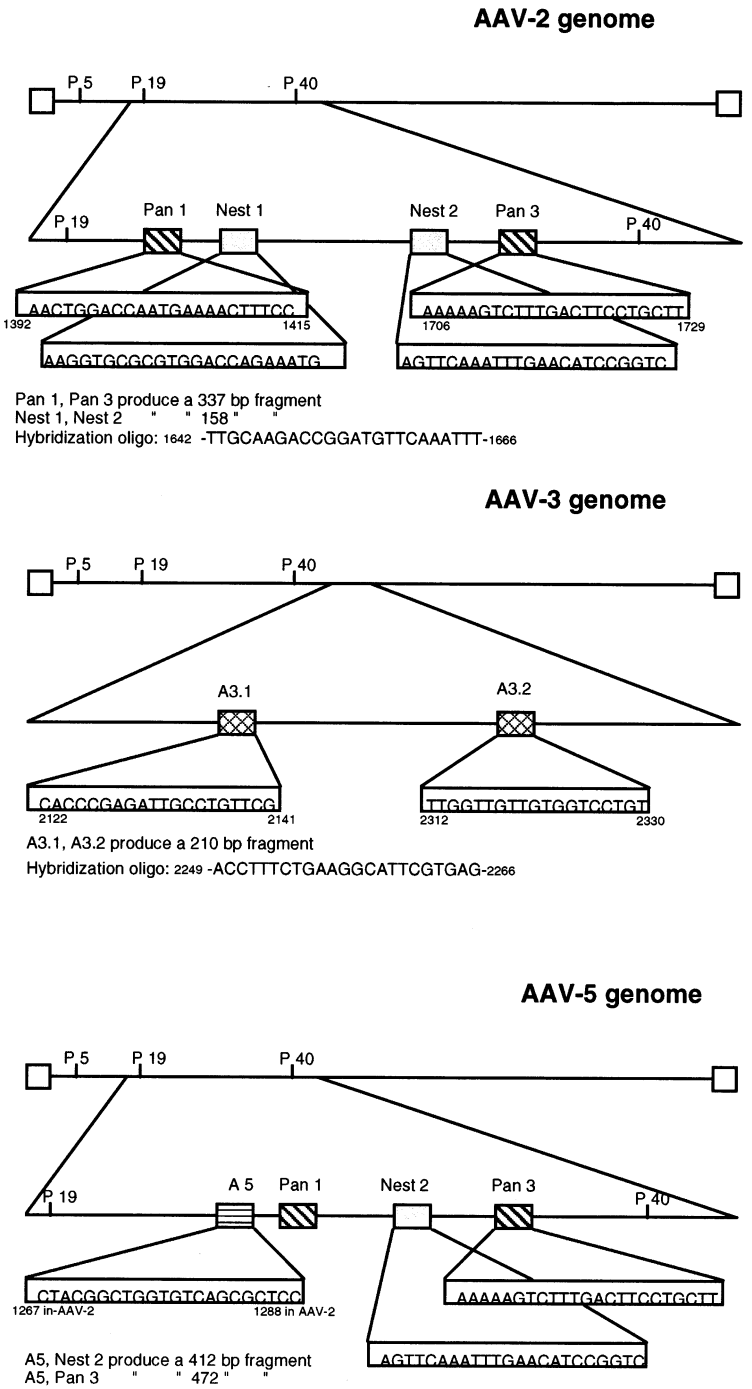

Fig. 3. Schematic representation of the primers, pan1, pan3, nest1, nest2, A3.1, A3.2 and A5 with respect to their nucleotide position within the genomic DNAs of different AAV types.

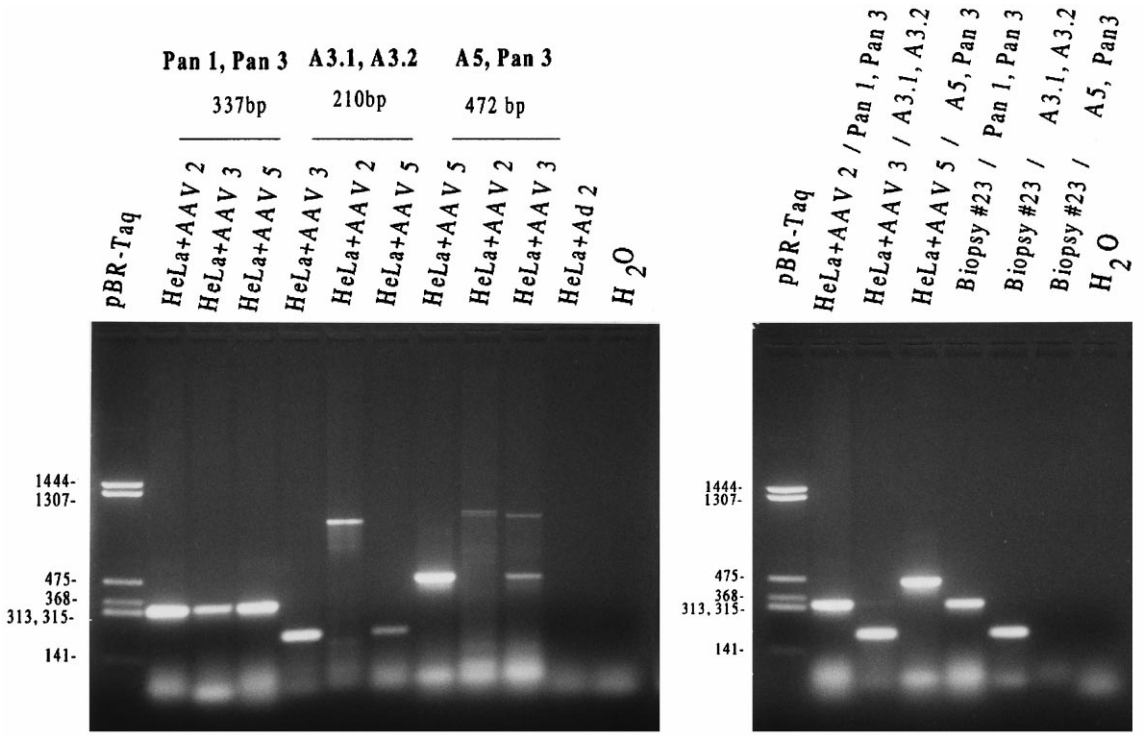

Fig. 4. Agarose gel analysis of PCR products from DNA of cells infected (m.o.i.: 100 p.f.u./cell) with AAV types 2, 3 or 5 (and co-infected with adenovirus type 2 (Ad2; m.o.i.: 10 p.f.u./cell)) and analysed with the primer pairs described in Fig. 3 (pan1, pan3, detecting all three AAV types; A3.1, A3.2, AAV-3-'specific' primers; A5, pan3, specifically hybridising to AAV-5 sequences). The lengths of the respective amplification products are indicated. DNA extraction was performed using the QIAamp® Kit (Hilden, Germany). The final 50 μl PCR mixture contained 1 μg DNA, 22 mM Tris–HCl (pH 8.4), 55 mM KCl, 1.65 mM $MgCl_2$, 220 mM deoxynucleoside triphosphates, 100 pmol of each primer and 1 U Taq DNA polymerase (GIBCO BRL, Eggenstein, Germany). The reaction mixtures were placed in a PTC-100™ (MJ Research) thermal cycler. The 'standard' PCR conditions were as follows: one initial denaturation step at 94°C for 2 min (once), then, 94°C for 2 min, 4 min at the annealing temperature of 62°C, 1 min at 72°C (elongation), for 35 cycles and one final elongation step at 72°C for 4 min. pBR322/Taq I (Appligene Oncor, Heidelberg, Germany) was used as the molecular weight marker. Thirty microlitres of each reaction were analysed for amplification products by agarose (1.5%) gel electrophoresis and ethidium bromide staining. (Using 'standard' conditions for all primers leads in some cases to additional, non-specific bands, but has the advantage of allowing type discrimination without using different protocols). As evident from the left hand panel, the pan1/pan3 primer pair amplify AAV sequences (337 bp fragment) from cells infected with either virus type. The AAV-3 specific primer pair, A3.1/A3.2 amplify DNA from AAV-3-infected cells (210 bp fragment), and to a lesser extent, also sequences from AAV-5-infected cells. However, the AAV-3 specific oligonucleotide described in Fig. 3 does not hybridise to the fragment amplified from AAV-5-infected cells (not shown). One AAV type 3 DNA containing clinical sample (biopsy # 23) is shown on the right hand panel as an example of human tissue containing this virus type (together with the respective controls). The fact that the input primers in the PCR of the biopsy using A5/pan3 are barely visible does not account for the failure to detect an amplified product, as was confirmed with experiments using higher amounts of DNA (not shown). In addition, the presence of AAV-3 DNA could be confirmed using the specific oligonucleotide probe mentioned above and described in Fig. 3 and in the text (data not shown).

with pan3 or nest2 as second primer. The amplified PCR products have a length of 472 or 412 bp, respectively.

Comparing the sequences of AAV types 2, 3 and 5, allowed to propose AAV type 3-specific primers (not detecting AAV-2 DNA) at nucleotide positions 2122–2141 (primer A3.1) and 2312–2330 (primer A3.2), respectively, giving rise to an amplification product of 210 bp (Fig. 3). A specific sequence to be used as hybridisation

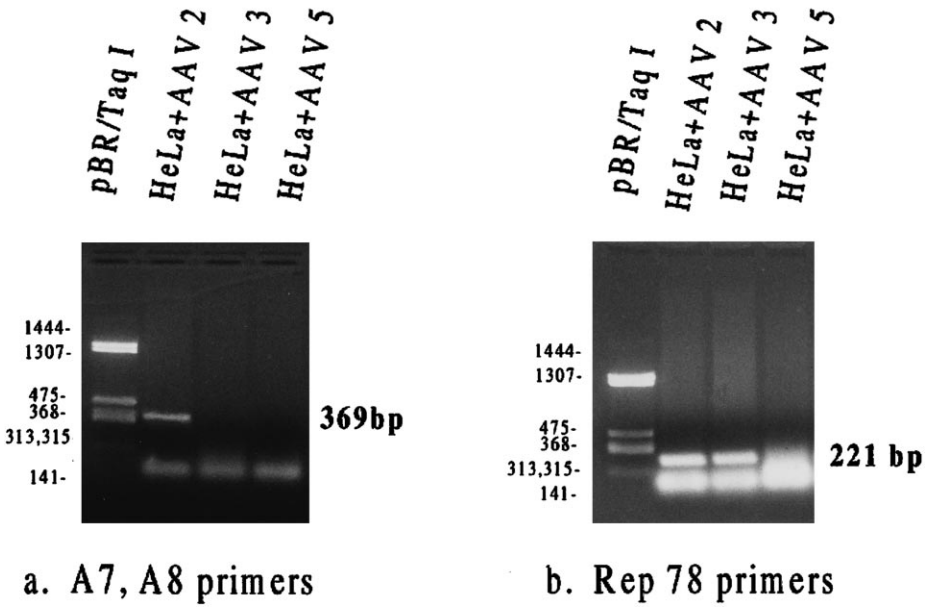

Fig. 5. Agarose gel analysis of PCR analyses of DNA from cells infected with AAV types 2, 3 and 5 (co-infected with adenovirus type 2 (Ad2)) as described in Fig. 4, and analysed with the primer pairs described by Han et al. (1996) (*a*. A7, A8) or Friedman-Einat et al. (1997) (*b*. Rep78), respectively. The size of the amplification products is indicated. pBR/Taq was used as a size marker. The primer pair A7/A8 amplified only DNA from AAV-2 (Panel a., 369 bp fragment), and the Rep78 primers were not able to distinguish between AAV-2 and -3, and did not amplify AAV-5 DNA sequences (Panel b., 221 bp fragment).

probe is described in Fig. 3. Since these primers detected also AAV-5 DNA it is probable that there is a high homology of type 5 and 3 sequences within the genome region coding for the capsid proteins (Fig. 4). However, the oligonucleotide specifically hybridising to amplified sequences of AAV-3 (Fig. 3) discriminates AAV-3 from AAV-5, i.e. it does not hybridise when the band visible in the gel is amplified from AAV-5 DNA.

The primer pairs proposed in Fig. 3 along with the type-specific oligo-nucleotide probes allow to determine and discriminate between the different AAV types present in tissue biopsies (Fig. 4). Primers pan1/pan3 detect all three types, and hybridisation with specific oligonucleotides can discriminate between types 2 and 5. A PCR using the A3.1/A3.2 primers (and the respective probe, Fig. 3) will detect AAV-3 and -5. In this case, an additional PCR using the AAV-5 specific primer, A5 in combination with nest2 or pan3 will allow to determine the correct type (Fig. 4). The primers A7/A8 described by Friedman-Einat et al. (1997) were found to detect only DNA of AAV-2, whereas the rep-78 primers proposed by Han et al. (1996) do not discriminate between AAV-2, -3, and will not amplify AAV-5 DNA sequences (Fig. 5).

In a series of studies with 30 different biopsies from human tissue which were found to contain AAV DNA, AAV type 3 was detected only in two cases (one example is given in Fig. 4), all others were identified as type 2. In no case was AAV-5 found. Albeit being preliminary because of the small number of samples, these data suggest that AAV-2 represents the most frequent AAV type infecting the human population.

The recent publication of the sequence of the genome of AAV type 4 (Chiorini et al., 1997) provides information for designing primer pairs specific for this virus type (e.g. the region between the nucleotide position around 3900–4075 in which the homology to types 2 and 3 is very low). However, AAV-4 is a simian virus and is not to be expected to be present in human samples (Murphy et al., 1995).

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