Aus dem Institut für Virologie, Campus Benjamin Franklin
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DISSERTATION

Prevalence and Characterisation of Adeno-Associated Virus (AAV) Persistent Infection in Leukocytes of Blood Donors and Immunosuppressed Patients

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von

Dina Khalid

aus Assiut, Ägypten

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Abstract

Adeno-associated virus (AAV) is ubiquitous but has not so far been associated with human disease. As a dependovirus, it has a biphasic AAV life cycle with latency phase and helper virus-induced productive replication phase. AAV *in vivo* persistence requires latency in specialized cell types to escape the host immune response until viral spread becomes possible. Reactivation from latency can be triggered by diverse stimuli including host immunosuppression. AAV has been thoroughly studied *in vitro*, but not *in vivo*. In view of the growing success of AAV-derived vectors in human gene therapy, it is becoming increasingly urgent to identify the *in vivo* target cells of wild-type AAV persistence to approach a better understanding of AAV *in vivo* biology.

We developed a highly sensitive and specific AAV PCR assay for the full spectrum of known human AAV serotypes. Using AAV PCR assay, we screened genomic DNA samples from leukocytes of 243 healthy blood donors (BD) and 41 immunosuppressed patients (IS). We observed that AAV is highly prevalent in human leukocytes, perceived as a potential reservoir for AAV latency. AAV-DNA was detected in about 34% and 76% of healthy blood donors and immunosuppressed patients, respectively. Mixed AAV infections were observed in 11% and 45% of BD and IS, respectively. Serotypes detected were similar in both groups, predominantly AAV2, followed by AAV5. Other serotypes such as AAV1, 3, 6, 8 and 9 were less commonly isolated. The high prevalence and broad spectrum of human AAVs in leukocytes as detected by PCR closely follows AAV seroepidemiology. The higher AAV detection rate and mixed infection in immunosuppressed patients are highly statistically significant, suggesting a possible reactivation of latent AAV infection. Many recent studies correlated immunosuppression with an increased frequency of AAV capsid-specific T-cells, implying a potential role of immunosuppression in AAV reactivation.

Some AAV-positive blood donors were repeatedly tested over a follow-up period of two years, and showed a repeated detection of the initial and/or additional AAV serotypes in many cases, suggesting persistent infection with fluctuating AAV viral load levels. Cloning of PCR products, where raw sequencing data showed superimposed peaks, confirmed our postulation of even an underestimated mixed infection using consensus primers and explaining the alternate detection of different AAV serotypes.

It was then interesting to identify the target leukocyte subpopulation for AAV persistence. Leukocyte separation revealed that AAV2 resided exclusively in $CD3^+$ T-lymphocytes,

considered as the plausible *in vivo* reservoir of AAV persistence. AAV5 was detected once, notably in both $CD3^+$ and $CD3^-$ fractions. Further experiments are required to further explore the leukocyte subpopulations for AAV persistence, as well as other human cells.

Zusammenfassung

Das Adeno-assoziierte Virus (AAV) ist allgegenwärtig, ist aber bisher mit keiner menschlichen Erkrankung in Verbindung gebracht worden. Als Dependovirus hat es einen zweiphasigen AAV-Lebenszyklus mit einer Latenzphase und einer Helfervirus-induzierten produktiven Replikationsphase. Die AAV *in vivo* Persistenz erfordert Latenz in spezialisierten Zelltypen, damit der Kontakt mit dem Immunsystem vermieden wird, bis die virale Ausbreitung möglich wird. Die Reaktivierung aus der Latenz kann durch verschiedene Stimuli einschließlich Host-Immunsuppression ausgelöst werden. AAV wurde *in vitro* eingehend untersucht, aber nicht *in vivo*. Im Hinblick auf den wachsenden Erfolg von AAV-abgeleiteten Vektoren in der humanen Gentherapie wird es immer dringender, die *in-vivo*-Zielzellen für die -AAV-Wildtyp-Persistenz zu identifizieren und besseres Verständnis für die AAV *in-vivo*-Biologie zu gewinnen/erlangen.

Wir entwickelten einen hochsensiblen und spezifischen AAV PCR-Assay, der eine Detektion des gesamten Spektrums der bekannten humanen AAV-Serotypen erlaubt. Mit diesem AAV PCR-Assay untersuchten wir 284 DNA-Proben aus Leukozyten, die von 243 gesunden Blutspendern und 41 immunsupprimierten Patienten stammten. Wir beobachteten eine deutliche Präsenz des AAVs in menschlichen Leukozyten, sodass sie möglicherweise als potentielles Reservoir für latente AAVs fungieren. Die AAV-DNA wurde in etwa 34% der gesunden Blutspender und 76% der immunsupprimierten Patienten detektiert. Zelluläre AAV-Infektionen mit mehreren AAV-Serotypen gleichzeitig wurden in 11% der Blutspender und 45% immunsupprimierten Patienten beobachtet. Überwiegend wurde das AAV-Serotyp 2 nachgewiesen, gefolgt von AAV5. All die anderen AAV-Serotypen wie AAV1, 3, 6, 8 und 9 kommen laut unserer Methode seltener vor.

Die erhöhte Präsenz der AAV Einzel- und Mischinfektionen bei den immunsupprimierten Patienten ist im Vergleich zu Leukozyten der gesunden Blutspender statistisch hoch signifikant, was auf eine mögliche Reaktivierung der latenten AAV-Infektion hinweist. Laut neueren Studien korreliert die Immunsuppression mit einer erhöhten Frequenz von AAV-Kapsid-spezifischen Zellen, was auf eine mögliche Rolle der Immunsuppression in AAV Reaktivierung hindeutet.

Die hohe Prävalenz bei der AAV-Präsenz und breites AAV Serotypen-Spektrum in menschlichen Leukozyten, die in dieser Arbeit durch den PCR-Assay nachgewiesen wurden, korreliert mit der beschriebenen AAV Seroepidemiologie.

Einige der AAV-positiven Blutspender wurden über einen Beobachtungszeitraum von zwei Jahren wiederholt untersucht. Dabei konnte der detektierte AAV-Serotyp erneut und in vielen Fällen ein zusätzlicher AAV-Serotyp nachgewiesen werden. Dies deutet auf eine persistierende AAV-Infektion hin, die allerdings einigen Schwankungen unterliegt. Des Weiteren wurden die klonierten PCR-Produkte auch unter Verwendung von Konsensus-Primern sequenziert, was unsere These von unterschätzten zellulären AAV-Mischinfektion bestätigt und eine Erklärung dafür gibt, warum in einigen Blutspendern verschiedene AAV-Serotypen zu unterschiedlichen Zeitpunkten detektiert wurden.

Als Nächstes identifizierten wir die Subzellen der Leukozyten Population für die AAV Persistenz. Die MACS Methode ergab, dass AAV2 ausschließlich in $CD3^+$ T-Lymphozyten residierte und lässt diese Subpopulation als *in vivo* Reservoir für das latente AAV2 vermuten. AAV5 wurde jedoch überraschend in beiden $CD3^+$ und $CD3^-$ Fraktionen nachgewiesen. Die Frage nach der Subpopulation der Leukozyten so wie andere menschlichen Zellen für die AAV-Persistenz bedarf jedoch weitere Untersuchungen.

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Abbreviations

I. Common Abbreviations

aa	Amino acids
AAP	Assembly Activating Protein
AAV	Adeno-associated virus
AAV1 - 13	Adeno-associated virus type 1-13
ACK	Ammonium-Chloride-Potassium buffer
Ad	Adenovirus
Ad5	Adenovirus type 5
ATCC	American Type Culture Collection
BSA	Bovine Serum Albumin
$Cacl_2$	Calcium chloride
cap	Capsid gene of AAV
CBA	Chicken ß-actin
CD	Cluster of differentiation
CF	Cystic fibrosis
CI	Confidence Interval
CMV	Human cytomegalovirus
CO_2	Carbon dioxide
CPE	Cytopathic effect
CTL	Cytotoxic T-lymphocytes
ddH_2O	Double-distilled water
DDs	Duplex dimers
DMEM	Dulbecco's modified Eagle medium
DMs	Duplex monomers
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid; ss- single stranded, ds- double stranded
DNase	Deoxyribonuclease
dNTPs	Deoxyribonucleoside triphosphates
E.coli	Escherichia coli
EBV	Epstein-Barr virus
EDTA	Ethylene - diamine - tetra - acetate
ELISA	Enzyme - linked immunoabsorbent assay
FCS	Foetal calf serum
FIX	Factor IX
h	Hour
H_2O	Water
HCC	Hepatocellular carcinoma

HEK293	Human Embryonic Kidney 293 cells
HeLa	Henrietta Lacks immortal cell line
HHV6	Herpesvirus type 6
HSPG	Heparan sulphate proteoglycan
HSV s	Herpes simplex virus
IBD	Inflammatory Bowel Diseases
ICP0 and ICP4	Infected Cell Polypeptide 0 and 4
IEE	Integration efficiency element
IgG(s)	Immunoglobulin G(s)
ITR(s)	Inverted terminal repeat(s)
IU	Infectious unit(s)
kDa	kilo Dalton
LB	Luria broth medium
m (g, l, m)	milli – (gram, litre, metre)
MACS	Magnetic-activated cell sorting
MOI	Multiplicity of infection
mRNA	messenger RNA
NA	Neutralizing antibodies
NEB	New England Biolabs
ng	Nanogram
NHP	Non‐human primate
nm	Nanometer
no	Number
nt	Nucleotide
Ø	Diameter
O/N	Over night
OH group	Hydroxyl group
ORF(s)	Open Reading Frame(s)
p	plasmid
p5/19/40	Map unit position 5/19/40 promoter
P5IEE	P5 integration efficiency element
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque Forming Unit
pH	power (Potenz) Hydrogen
PLA2	Phospholipase A2
qPCR	quantitative real‐time PCR
rAAV	recombinant AAV
RBCs	Red Blood Cells
RBE	Rep Binding Element
RBS	Rep Binding Site
rep	non‐structural gene of AAV

RNA	Ribonucleic acid
RNase A	ribonuclease
RPMI 1640	Roswell Park Memorial Institute 1640 medium
RT	Room temperature
s	Second(s)
scAAV	self‐complementary AAV
SDS	Sodium dodecyl sulphate
sf9	Clonal isolate of Spodoptera frugiperda Sf21 cells
ss	single‐stranded
TAE	Tris‐Acetate‐EDTA
TE	Tris‐EDTA
TNNT1	Skeletal muscle troponin T gene
trs	Terminal resolution site
U	Unit(s)
UV	Ultraviolet light
v/v	volume/volume
VP	Structural proteins of AAV
VZV	Varicella zoster virus
w/v	weight/volume
wt	wild‐type

II. Units and Physical Scales

(n) x g	n times gravitational acceleration
°C	Degree Celsius
bp	Base pairs
D	Dalton
g	Gram
h	Hour
kb	Thousand base pairs
l	Litre
M	Molar, mol/l
m	Meter
min	Minute
mM	millimolar
OD	Optical density
rpm	Revolutions per minute
s	Second
U	Units
V	Volt
Vol.	Volume
W	Watt

III. Prefix of Scale Units

k	Kilo	10^{3}
m	Milli	10^{-3}
μ	Micro	10^{-6}
n	Nano	10^{-9}
p	Pico	10^{-12}

IV. One and Three Letter Code of the Amino Acids

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartate
E	Glu	Glutamate
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asp	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

1 Introduction

1.1 Adeno-Associated Virus Biology

1.1.1 Taxonomy

Adeno-associated virus (AAV) was first described almost seventy years ago as a contaminant of human or simian adenovirus (Ad) stocks, from which its name is derived (1-4). AAV is a small (20 nm), non-enveloped single-stranded DNA virus, with an icosahedral capsid (Figure 1.1). It belongs to the family Parvoviridae, genus Dependovirus (5, 6). AAV is a defective virus that needs a helper virus for active replication, such as adenovirus (1) and several herpesvirus genera, including herpes simplex virus (HSV) (7), varicella zoster virus (VZV) (8), human cytomegalovirus (HCMV) (8, 9), Epstein-Barr virus (EBV) (8), and human herpesvirus type 6 (HHV6) (10), all of which have been shown to support AAV replication. Human papillomavirus 16 (11) and vaccinia virus (12) have also been reported to provide AAV helper function.

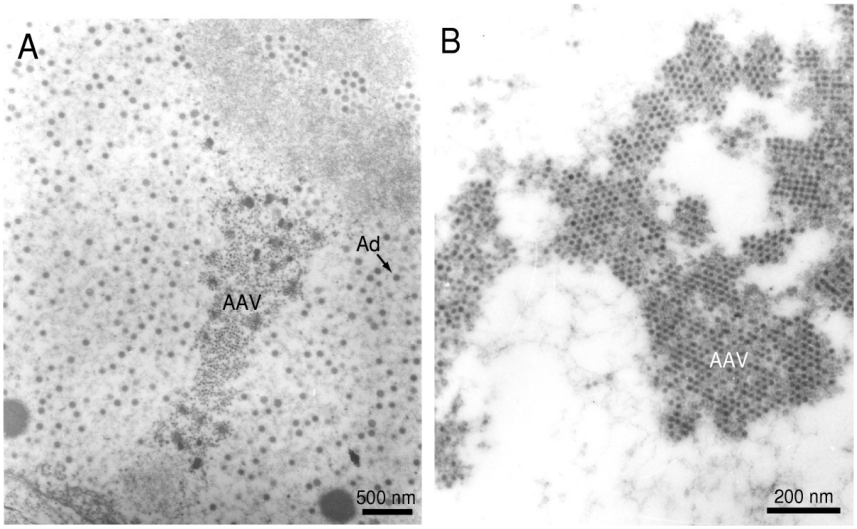


Figure 1.1 Transmission Electron Microscopy of AAV2 and AD5 particles in human cells.

A. AAV2 and Ad5 particles in the nucleus of a HeLa cell at 48 hours after co-infection. Magnification: × 15,000. **B.** AAV2 virions in a HeLa cell at 48 hours after co-infection with Ad5. Magnification: × 40,000. The figure and legend are taken from Goncalves, 2005 (13).

1.1.2 AAV Genome

AAV packages a linear single-stranded (ssDNA) genome of about 4.7 kb (14). The sense and antisense ssDNA strands are packaged with equivalent frequency (15, 16). The AAV genome consists of two genes: *rep* encoding the synthesis of non-structural proteins (17, 18), and *cap* encoding the synthesis of structural proteins (19, 20). Four non-structural proteins are described: rep78, rep68, rep52, and rep40 (17, 21, 18, 22, 23). The synthesis of rep78 and rep68 is induced by the p5 promoter; while that of rep52 and rep40 is under the control of the p19 promoter (24, 25). Rep controls almost every phase of the AAV life cycle and has been reported to play an essential role in site-specific integration *in vitro* (26-34). There are three structural proteins: VP1, VP2, and VP3, whose translation is initiated by the p40 promoter, and which are differentially produced by alternative splicing and different start codons (35, 36, 29). These three promoters have been mapped and named according to their map position (37). Both *rep* and *cap* genes are flanked on either side by inverted terminal repeats (ITR). ITRs are *cis*-acting 145 bp sequences that play a major role in the downregulation of virus replication in non-permissive conditions, provide an origin for replication in permissive conditions, support rescue of the virus and site-specific integration, and are essential for packaging the replicating virus (38-40, 21, 18, 26, 41-43) (Figure 1.2). The first 125 bp of the ITR folds on itself during replication and forms a palindromic T-shaped sequence, which has two independent orientations, flip and flop, according to whether the (B-B`) or (C-C`) subregion is closer to the 3` end, respectively (39, 44). The Rep binding site (RBS) and replication-dependent terminal resolution site (trs) are located on the stem palindrome (45). The remaining 20 nucleotides at the inner end of the ITR (D-D`) stay unpaired (Figure 1.2).

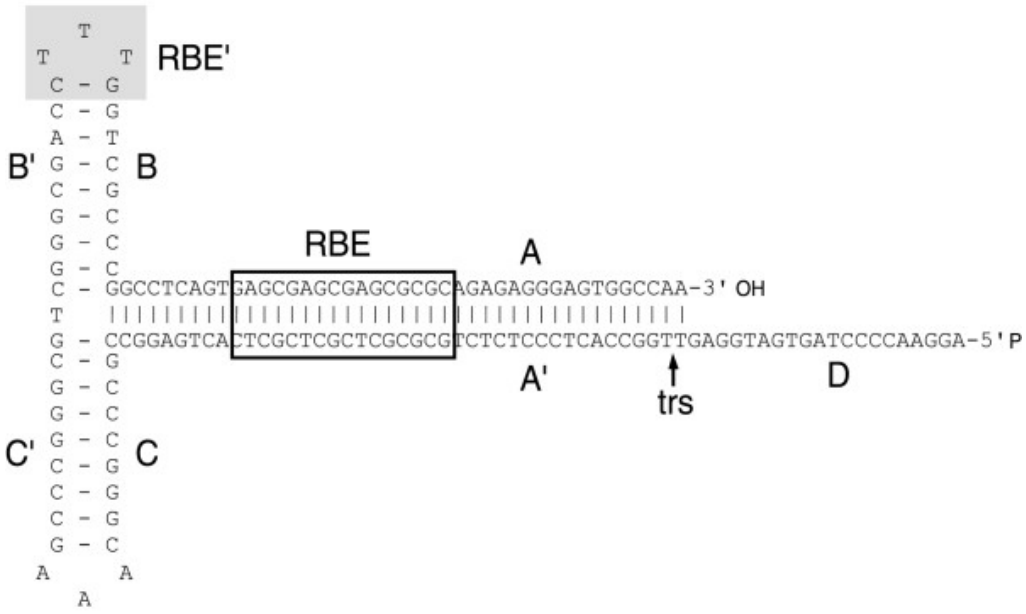


Figure 1.2 Secondary structure of the AAV2 ITR.

The AAV2 ITR is configured as a hairpin palindrome, with two arm palindromes (B-B' and C-C') within a larger stem palindrome (A-A'). The ITR can acquire two configurations (flip (depicted) and flop), depending on whether the B-B' or C-C' palindrome is closer to the 3' end, respectively. The D sequence is a single-stranded 20-nucleotide sequence at the end of the ITR. The boxed motif corresponds to the Rep binding element (RBE), while the grey-shaded nucleotides at the apex of the B-B' palindrome arm represent an additional RBE (RBE'). (RBE, GAGCGAGCGAGCGCGC; RBE`, CTTTG; and the TRS GGTTG). The figure and legend are taken from Daya and Berns, 2008 (34).

1.1.3 AAV Proteins

1.1.3.1 Non-Structural Proteins

Four non-structural proteins are described: rep78, rep68, rep52 and rep40, that are named according to their molecular weight as described before (17, 21, 18, 22, 23)(see 1.1.2). Each of them has ATPase and helicase functions, with 3`-5` polarity (46, 31, 47-50). Helicase activity of Rep78/68 mediates strand displacement so the new strands can be used for further replication or be packaged (51, 46, 31), while that of Rep52/40 helps encapsidation of viral genomes (51, 52). Rep78/68 binds to Rep binding site (RBS) and cleaves site specifically at the terminal resolution site (trs) after nucleotide (*nt*) 124, to unwind the hairpin and initiates viral replication (45, 53, 31, 32, 54, 49).

1.1.3.2 Structural Proteins

The capsid is composed of three proteins VP1, VP2 and VP3, of molecular weights 87 kD, 73 kD and 62 kD, respectively. Their production is induced by the p40 promoter, though at different frequencies (1:1:10) as a result of alternative splicing and different start codons (20, 35, 36, 29). Different functional domains have been described for capsid proteins. The first one characterized belongs to VP3 (55, 56) and is located on the capsid's outer surface (57). It is responsible for Heparan sulphate proteoglycan (HSPG) binding, facilitating cell attachment and subsequently infection (58). Another domain binds to the α5β1/αVβ1 integrin, which acts as an alternative coreceptor for virus cell entry (59). Lastly, the phospholipase A2 domain (PLA2), like most other parvoviruses, in the VP1 is believed to play an essential role in virus infectivity (60, 61). An alternative open reading frame of the *cap* gene codes for the assembly-activating protein (AAP), so called as it plays a major role in capsid assembly through interaction with VP proteins (62-64) (Figure 1.3).

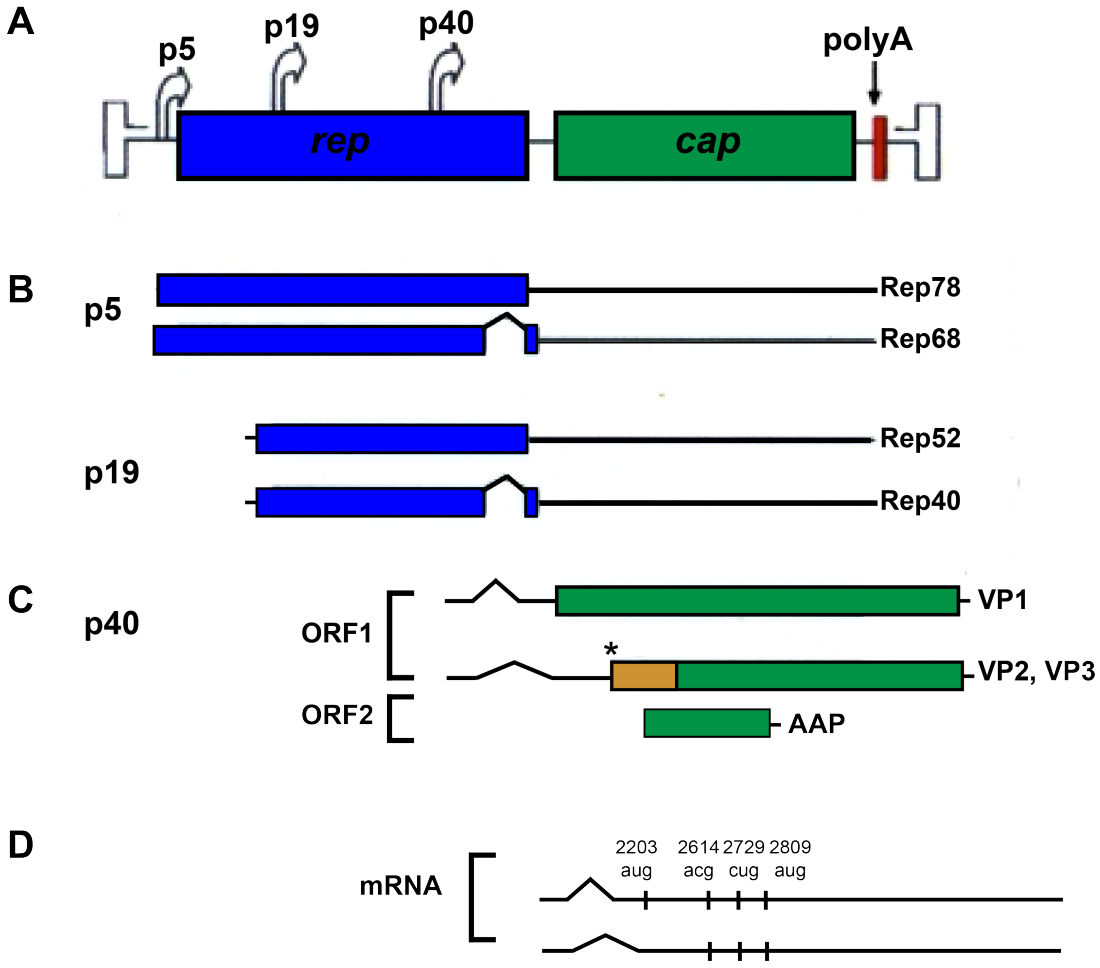


Figure 1.3 Genomic map of wild-type AAV2.

A. *Rep* and *cap* genes flanked by ITRs. The AAV genome contains three open reading frames (ORFs). The different Rep and Cap transcripts are produced from their respective promoters (P5, P19, and P40). **B.** The *rep* ORF (blue) encodes four Rep proteins by alternative splicing. **C.** The *cap* ORF (green) encodes three structural Cap proteins by alternative splicing of the mRNA transcript initiated by the p40 promoter. The asterisk indicates the alternative ACG codon used to produce VP3. The spliced mRNA that codes for VP3 from a conventional AUG start codon also codes for the VP2 protein, which has additional N-terminal residues (orange), from an upstream ACG start codon (asterisk). **D.** The translation initiation codons used for expression of the four proteins involved in AAV2 capsid formation (VP1, VP2, VP3, and AAP) are indicated. Only the minor splice product contains the translation initiation codon for VP1. The novel assembly-activating protein (AAP) is encoded by a nested, alternative ORF comprising a nonconventional CUG translation initiation codon. The figure and legend are adapted from Daya and Berns 2008 (34), and Sonntag et al. 2010 (62).

1.1.4 AAV Replication

The currently accepted model for AAV replication is based on self-priming and single-strand displacement synthesis. ITR supports AAV replication by acting as a primer for second DNA strand synthesis. The parental strand is nicked within a 7-base recognition sequence called the terminal resolution site (trs), supplying a free 3´ OH group, which allows DNA polymerase to unwind and synthesize new ITR. Via single-strand displacement synthesis, the linear duplex termini are reconfigured into terminal hairpin palindromes. Then, newly synthesized single-stranded AAV genomes and replicative forms (duplex monomers, DMs) are generated. If nicking fails, duplex dimers (DDs) with head-head or tail-tail configuration are formed (65, 13, 66) (Figure 1.4).

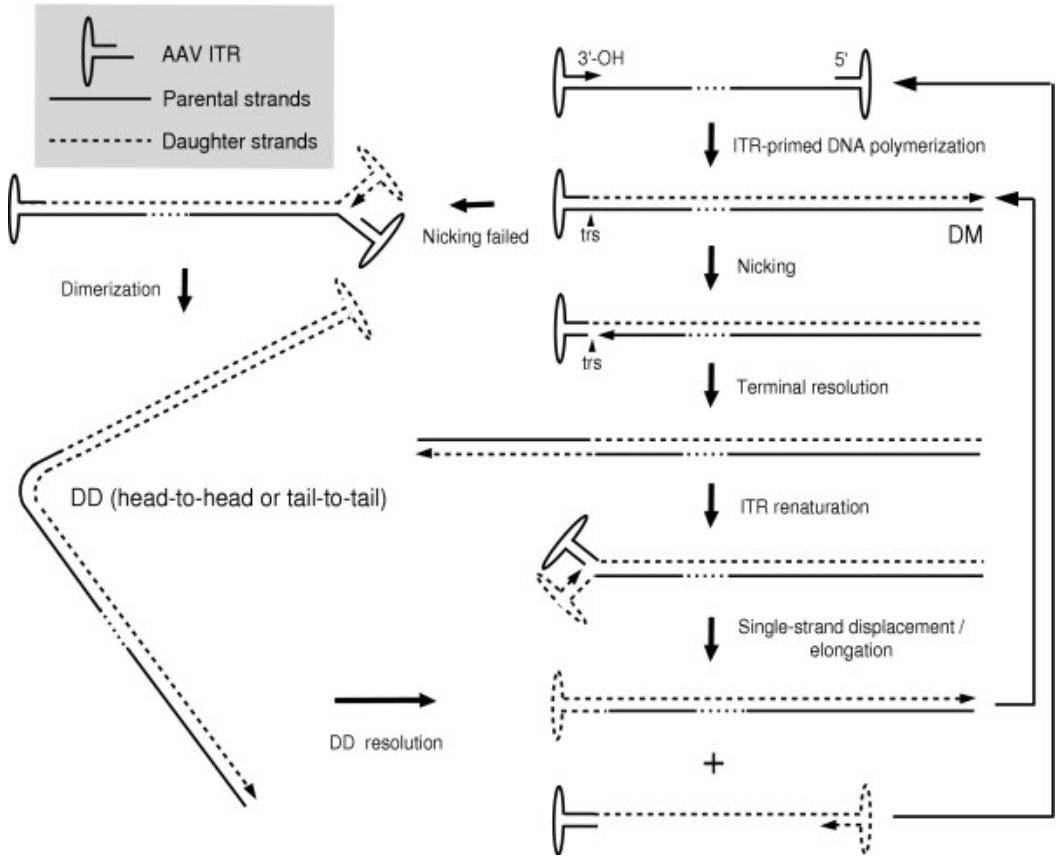


Figure 1.4 AAV-DNA replication.

ITRs provide the origin for AAV replication, where DNA polymerizes at the 3´ OH end of the ITR, forming replicative forms called duplex monomer DMs. The parental strand is nicked at the terminal resolution site (trs), providing a free 3´ OH end for DNA polymerase to unwind and synthesize a new copy of the ITR. The terminal palindromic sequences now configure as hairpin structures allowing single-strand displacement synthesis. If nicking fails, double-length double-stranded molecules, duplex dimers (DDs), are generated with head-to-head or tail-to-tail configuration. The figure is taken from Goncalves, 2005 (13). The legend is adapted from (65, 13).

1.1.5 AAV Life Cycle

AAV is unique in the fact that it establishes a latent infection in the absence of helper virus (67), where the viral genome is uncoated and remains either as an integrated provirus (68-70) or an episome (69, 71) in *in vitro* studies. Early studies indicated that wild-type AAV integrates site-specifically by non-homologous recombination, mostly at a specific locus termed AAVS1 on chromosome 19q13.42 (72-74, 70). The AAVS1 locus is located in gene "MBS85" responsible for actin biological regulation (75), and just near to another skeletal muscle troponin T gene, TNNT1 (76), whereby AAV integration might disrupt the function of these genes. A roughly 8.2 kb DNA sequence including the specific site of integration on chromosome 19 was analysed, and it seemed initially that the 5` terminal 512 nucleotides are essential for targeted integration (74, 77). ITR, 16 bases of RBE_{p5} (the so-called, P5 integration efficiency element, P5IEE) (both *in cis*) and Rep 78/68 (*in trans*) are required for targeted integration (73, 78, 79). However, recent studies have reported that wild-type AAV integrates in HeLa cells and human fibroblasts with preference for Rep-binding site homologs, which are scattered over the entire human genome, including hotspots on chromosomes 1, 3, 5, and 19 and suggested that the integration profile is cell-line specific and dependent on chromatin accessibility (80, 81). *In vivo*, the situation is much less clear, where AAV tends to persist as an episome rather than to integrate (82-84). Some concerns have been raised about the potential mutagenic consequences of unspecific integration in neonatal mice (85-87), though other groups did not observe supporting evidence (88-90). Recently, AAV2-induced insertional mutagenesis was observed in HCC patients, especially in non-cirrhotic cases, though at a small proportion 5% (91), yet questioning the previous belief of AAV non-pathogenicity.

The other intercellular phase of AAV life cycle is a productive infection, mostly in the presence of a helper virus (1, 92, 7). Most of the helper functions supplied by AAV helper viruses have been characterized. All adenovirus (Ad) early genes; E1a, E1b, E2a, E4) and VA RNA are required for AAV replication (93-97), while in the case of HSV1, not only the genes involved in gene expression regulation (ICP0 and ICP4) are needed, but also viral DNA polymerase and helicase-primase complex (UL5, UL8, and UL52 proteins) (98-103) (Figure 1.5). However, AAV is not completely defective. Even in the absence of helper virus, certain mammalian cell lines, when exposed to stress or genotoxicity, as in the case of exposure to UV radiation, γ-radiation, genotoxic stress or carcinogens, allow the replication of AAV, albeit at much lower efficiency (12, 104-106). Many AAV helper viruses, e.g. Ad, CMV, EBV, and HHV6, also have a biphasic life cycle, where they persist latently in cells of

hematopoietic origin (107-111) and are reactivated upon immunosuppression with poorer prognosis (112-115).

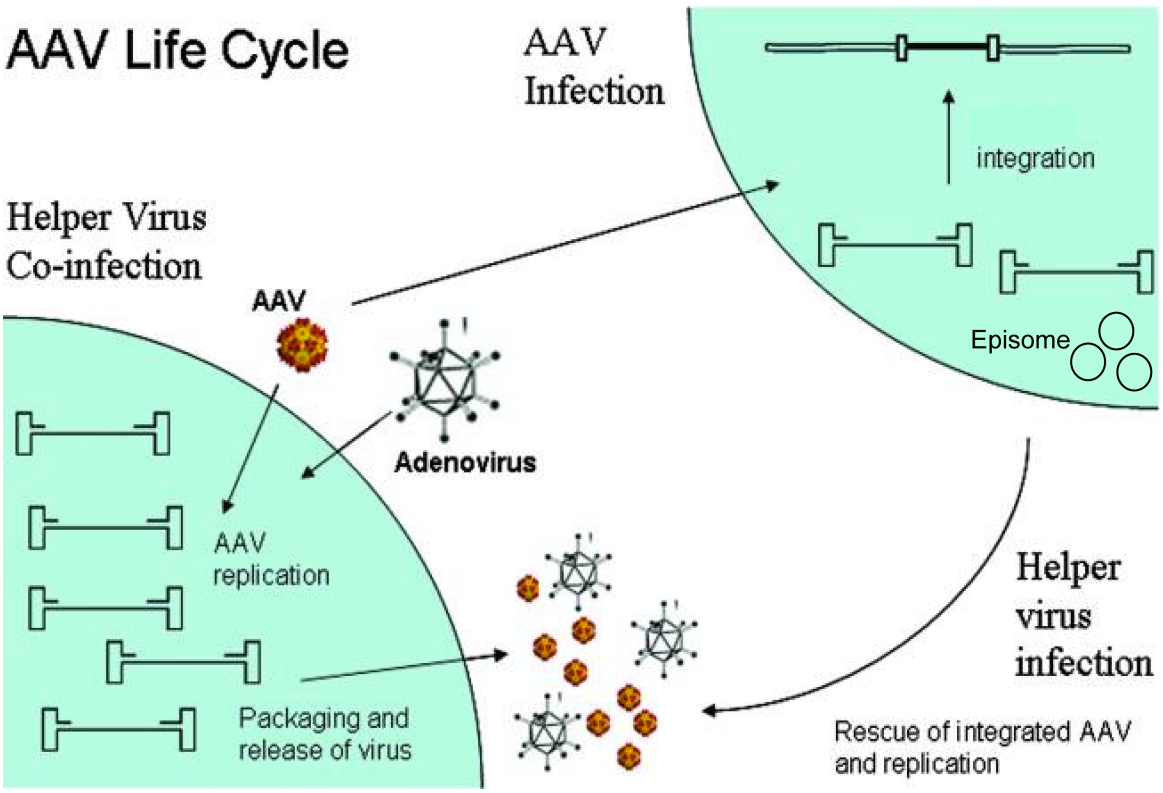


Figure 1.5 AAV life cycle.

In the presence of a helper virus, e.g. adenovirus, AAV replicates and undergoes a productive infection. In the absence of helper virus, AAV remains latent and either integrates or remains as an episome. AAV can be rescued from the latent stage upon helper virus superinfection. This figure and legend are modified from Daya and Berns, 2008 (34).

1.1.6 AAV Serotypes

AAV is widely disseminated and found in almost all species (116-122). So far, more than 100 human and non-human primate (NHP) AAV variants have been described. AAV isolates are subdivided into serotypes based on serology. Thirteen human/ NHP AAV serotypes have been identified, and most of their sequences have been described and share between 65% and 99% sequence identity (1, 123, 14, 124, 8, 125, 126, 3, 127-129, 117, 130-133). AAV serotype 1, 2, 3 and 6 were isolated initially as contaminants of adenovirus stocks (1, 125, 3). AAV2 is the first serotype cloned and thoroughly studied and is considered to be the prototype of the human AAV (134, 135). Neither AAV4 DNA, nor antibodies reactive against it could be detected in human so far, but only reported in non-human primates (136, 137, 126, 130). AAV5 and 9 have been directly isolated from human tissues (8, 130). AAV5 is the most divergent human serotype described so far, having only ~ 60% homology with other AAV serotypes and has been suggested to be transmitted sexually along with herpes simplex virus (HSV) infection (124, 8, 128). AAV8 has been isolated from both human and non-human primate tissues (117, 130), while AAV7, 10 and 11 have till now only been detected in non-human primate tissues (130, 131). AAV12 and 13 have been isolated as contaminants of simian adenovirus stocks (132, 133). Another method for the classification of AAV isolates into clades is based on phylogenetic relatedness (Figure 1.6). PCR-based screening revealed the broad distribution of many new AAV isolates in human as well as non-human primates (NHP) and virus stocks (130, 138, 139). Six human AAV clades (A-F) and two clonal isolates (AAV4, and 5) have been described using computational analysis on a wide range of isolated primate AAV sequences (130). Another less traditional and rather more sophisticated method for AAV subgrouping is the transcapsidation method, which classifies AAV isolates based on their ability to form unique cross-dressed virions, and functionally delineates their capsid structural relatedness (140).

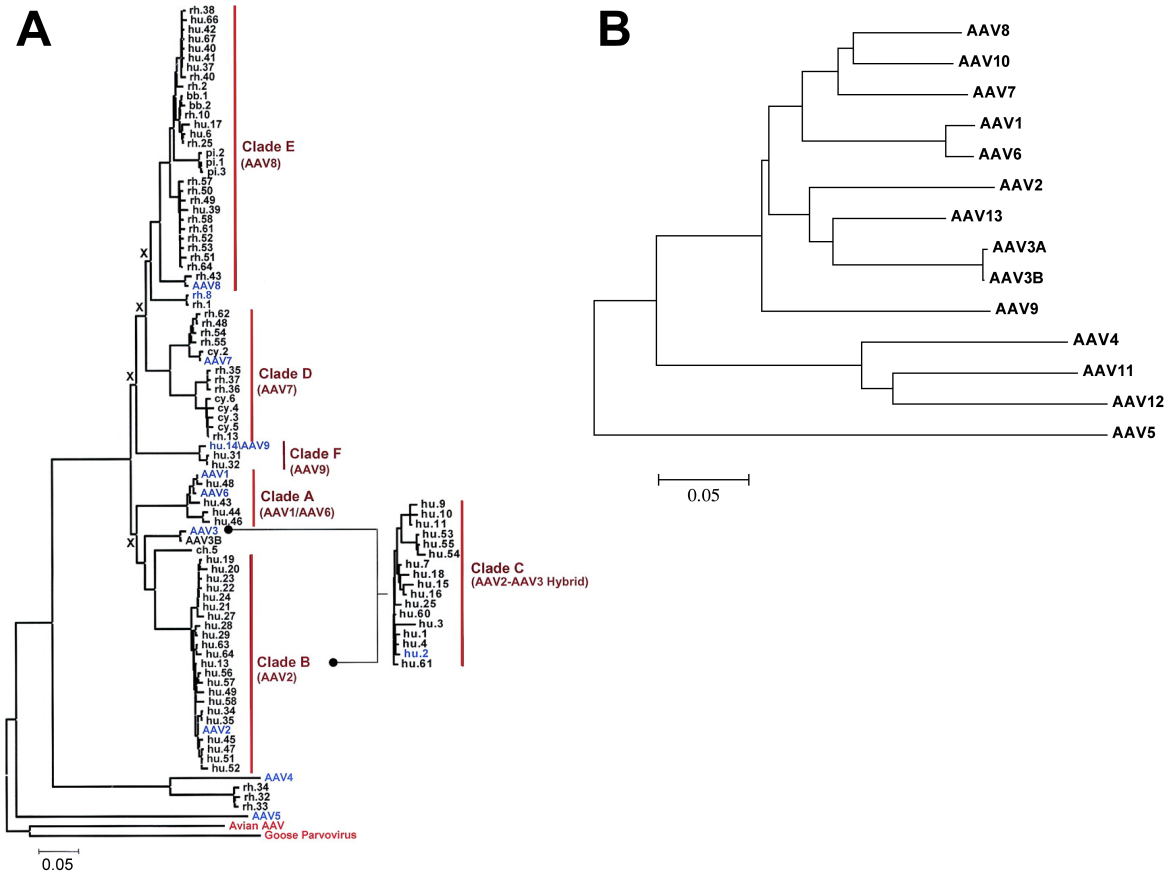


Figure 1.6 Neighbour-joining phylogenic tree of AAV isolates.

A. Neighbor-joining phylogenies of the VP1 protein sequence of primate AAVs generated using MEGA v2.1 and TreePuzzle packages. Clades are indicated by name and by red vertical lines to the right of their taxa. The nomenclature for the taxa is either the serotype name or a reference to the species source (hu, human; rh, rhesus macaque; cy, cynomolgus macaque; bb, baboon; pi, pigtailed macaque; ch, chimpanzee), followed by a number according to the order in which they were sequenced. Figure A is taken and legend A is modified from Gao et al. 2004 (130). **B.** Using MEGA v6.06, a neighbor-joining phylogenetic tree of the known sequences of AAV serotypes 1-13 was generated to show the relatedness of these serotypes.

1.1.7 AAV Seroepidemiology

Early studies reported that 30-80% of the human population displayed antibodies against the human AAV serotypes 1–3 and AAV5 (136, 141-143, 8). Direct comparison of serotype-specific IgG showed a seroprevalence for AAV1 and AAV2 of around 70%, whereas seroprevalences for AAV5 (40%), AAV6 (46%), AAV8 (38%) and AAV9 (47%) were generally lower (144). The development of neutralizing antibodies against AAV1, 2, 5 and 8

followed the same pattern (144, 145). In almost all studies, anti-AAV2 antibodies scored the highest prevalence and the highest titre in the case of co-prevalence, and cross-reaction seems to play an important role (146, 144, 147). While some studies claimed no significant difference in the geographical distribution of AAV2 seroprevalence (148), others recently reported a significantly different distribution of neutralizing antibodies (NA) against AAV1 in heart failure patients between Europe and the USA (67.7% vs. 53.5%; p <0.0001) (147). Moreover, a difference in the distribution of NA against AAV1, 2, 7 and 8 in four continents was noticed, with Africa recording the highest NA prevalence for the four serotypes tested, though the relative frequency of NA against different serotypes was consistent (146). Lack of a standardized method might explain the differences. AAV antibodies could be detected in newborns, suggesting vertical transmission of the virus or, more likely, passive immunity as the antibodies decreased gradually by the age of 7-11 months, then slowly increased again by the age of one year through adolescence (149), and showed a significantly steady increase with age (148, 147). There is some controversy about sex predilection of AAV seroprevalence (148, 150, 147). Anti-AAV2 IgG antibodies increased significantly in pregnant women, compared with the control group, though no increase in anti-AAV2 IgM was observed (148).

1.1.8 AAV Pathogenicity

AAV is generally considered apathogenic, as no direct correlation with human disease could be drawn with AAV infection. Some controversy had been raised, however, as some studies have proposed that AAV is correlated with adverse reproductive outcomes such as abortion, preeclampsia, amniorrhexis, premature labour and placental dysfunction (151-155), while others have postulated that AAV has tumour suppressive activities (156-159). One major concern is that rAAV insertional mutagenesis has been associated with a considerable risk of developing hepatocellular carcinoma (HCC) in neonatal mice (86), though it was suggested that this correlation more likely relates to neonatal mouse vulnerability to cancer rather than to rAAV-induced mutagenesis (88, 89, 87, 90). Recently, clonal integration of AAV2 in cancer driver genes was observed in 6% of human HCC biopsies (160, 91), mandating more information to better interpret a possible correlation.

1.1.9 AAV in Human Host

Although cell receptors have been identified for many AAV serotypes, and susceptible cell types have been described, little is known about the primary routes of AAV infection (161).

Only a few studies have been carried out to address the AAV *in vivo* life cycle (83, 130, 84). Most knowledge about AAV is drawn from *in vitro* studies. The ubiquitous presence of AAV notwithstanding, the mode of spread and the preferential cells for its persistence are not known. AAV is presumed to spread through the respiratory tract along with adenovirus infection, supported by the observation of seroconversion in early childhood closely following that of adenovirus (141, 149). This is not the case for the most divergent serotype, AAV5, where seroconversion was reported to occur later around the age of 15-20 (8) following that of herpes simplex virus (HSV), and giving rise to the assumption of sexual transmission. Sexual transmission was also suggested by the retrieval of AAV-DNA from genital tissues, semen, and abortion material (151, 162, 163, 152, 164, 153). From the primary portal of entry, it seems that AAV may spread via the bloodstream to distant locations, either as a free virus or carried in blood cells; i.e. leukocytes. AAV-DNA has been readily found at the site of inoculation and in peripheral blood mononuclear cells (PBMCs) in a subset of macaques iatrogenically infected with AAV alone or with adenovirus (83). Consistent with the notion of hematogenous spread is the detection of different AAV clades in almost every human and non-human primate tissues tested (130).

1.2 AAV as Gene Therapy Vector

1.2.1 Why is AAV a Promising Gene Therapy Vector?

Widespread acceptance and interest in AAV as a gene therapy vector has many aspects. To date, AAV could not be proved to be a causative agent of any human disease. Besides, AAV is less likely to cause detrimental cellular immune response than adenovirus and has not been reported to cause toxicity or strong destructive immune response in animal models (33, 165-167). AAV serotypes also have a broad, diverse tropism and ability to transduce diverse tissues and cell types, including lung tissues (168, 169), hepatocytes (170), muscle fibers (171), hematopoietic progenitor cells (172-176), lymphocytes (177), melanoma cell lines (178), keratinocytes (179), and even the gut epithelial and lamina propria cells (180) (for details of the tropisms of different serotypes please refer to Luo et al., 2015 (181)). A tremendous advantage of rAAV vectors in comparison to other gene therapy vectors is the ability to transduce non-dividing cells (182-184). A wide range of genes has been expressed using rAAV vectors, including immune-directed gene therapy, cancer gene therapy, genetic and metabolic diseases, and even vaccine generation (185, 34, 181). Long-term transgene expression in different animal models has been reported (186-191).

AAV is a very promising gene therapy vector and is increasingly attracting interest, with

more encouraging clinical trials results. The first approved commercially available human gene therapy, Glybera, is a recombinant AAV vector (rAAV) encoding lipoprotein lipase to treat metabolic lipid disorders (192). The establishment of the first infectious clone of AAV2 in 1982 enabled the study of AAV2 genetics and its *in vitro* life cycle and provided a basis for rAAV (134). The first rAAV vectors were produced shortly after that (193, 135, 194). About ten years later, the first clinical trial with rAAV was carried out (195). Recombinant AAV vectors (rAAV) consist of a transgene (therapeutic gene) flanked on either side by ITR. They are devoid of *rep* and *cap* genes, which were replaced with the transgene of interest, as ITRs contain all the elements needed for replication, packaging, and rescue *in cis*, (see section 1.1.5). Rep and Cap expression cassettes were provided *in trans* either as helper plasmids or in stable cell lines expressing them, in addition to adenoviral helper functions (196-200). A recent scalable alternative is the use of insect cell line *sf9* and recombinant baculovirus plasmids (Bac) encoding rAAV, Rep and Cap (201-203). Recently, a new unified baculovirus-based rAAV production system for AAV1-12 “OneBac” was described, which consists of a stable *sf9* cell line with integrated rather silent “AAV1-12“ *rep* and *cap* under the control of the chicken ß-actin-CMV hybrid (CBA) (204, 205). (Figure 1.7)

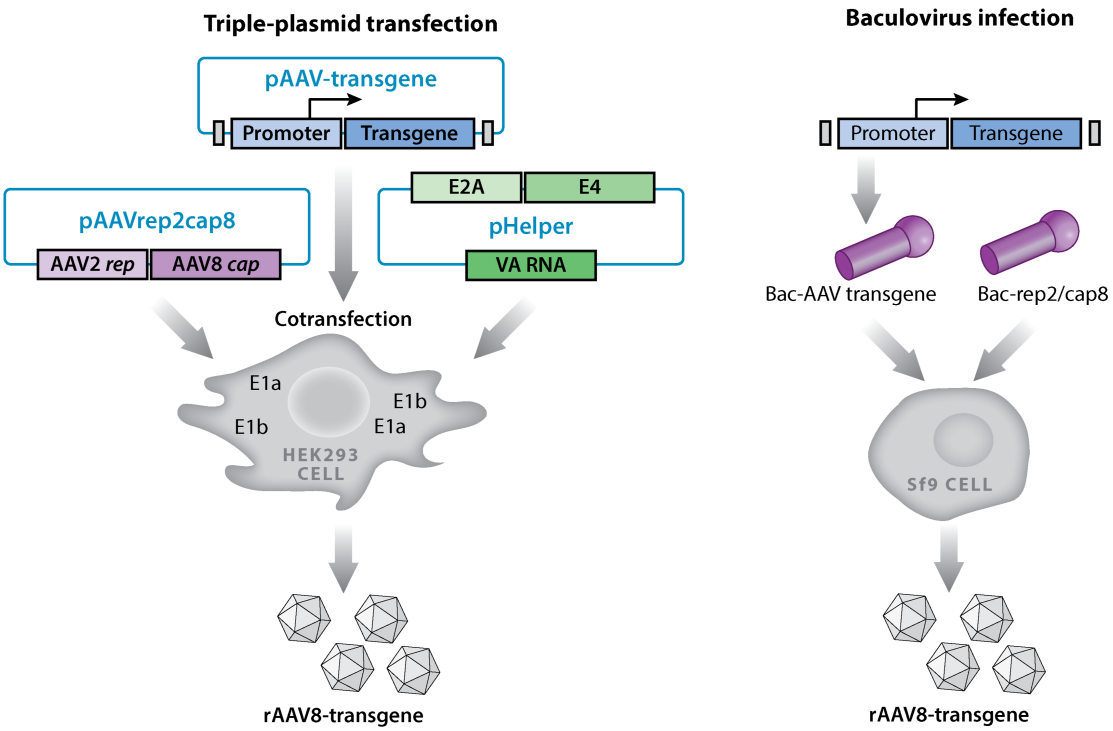


Figure 1.7 rAAV production methods.

In the triple-plasmid method, HEK293 cells, expressing adenovirus genes: E1a and E1b, are cotransfected with an adenovirus helper plasmid (pHelper), a rep/cap plasmid expressing AAV2 rep and AAV8 cap (pAAVrep2cap8), and the transgene plasmid carrying the rAAV-transgene cassette (pAAV-transgene). In the baculovirus approach, the rAAV-transgene cassette is built into a baculovirus, which is then used to infect *sf9* insect cells that are coinfected with a second baculovirus expressing *rep2* and *cap8* under the control of baculovirus promoters. Both the baculovirus and plasmid transfection methods produce rAAV expressing the transgene, but the baculovirus method (and similar herpes virus methods) typically produces 100 times more viruses per cell and is more easily scaled to large volumes of cells. The figure and legend are taken from Samulski and Muzyczka, 2014 (66).

1.2.2 Safety with Respect to Current Knowledge

Though the increasing acceptance and interest in AAV as a recombinant gene therapy vector, AAV gene therapy has some shortcomings and drawbacks that need more comprehensive approaches to understand and to overcome. Transgene size is limited to 4.7 kb and 10% increase is the maximum for encapsidation. Also, there is a lag after the introduction of AAV gene therapy until expression of the therapeutic gene for second-strand synthesis (206). Self-

complementary AAV vectors (scAAV) have been designed to bypass the lag for complementary-strand synthesis and to increase the rAAV transduction (207, 208). However, this approach decreases rAAV packaging capacity by 50% (~2.4 kb), though up to 3.3 kb DNA can still be encapsidated (209). A trans-splicing recombinant AAV vector has been developed to increase the packaging capacity of rAAV to harbour larger genes (210) (Figure 1.8). Therapeutic genes of up to 9 kb can be delivered in different tissues (211-213), but it is less efficient than conventional rAAV (34). Moreover, site-specific integration is one of the most controversial aspects of AAV life cycle. As rAAV vectors do not encode Rep and lack the integration efficiency element (IEE)/RBE in *cis*, they do not integrate and remain mainly extrachromosomal (82, 214-217, 184). The consequences are controversial; some researchers think it is advantageous in terms of safety if rAAV persists as an episome, and does not integrate, while others believe that loss of targeted integration might have an adverse impact on the long-term expression of rAAV (82, 66). More knowledge of the *in vivo* biology of AAV would provide a better understanding and helps to develop better approaches for a safe and efficient AAV-mediated gene therapy.

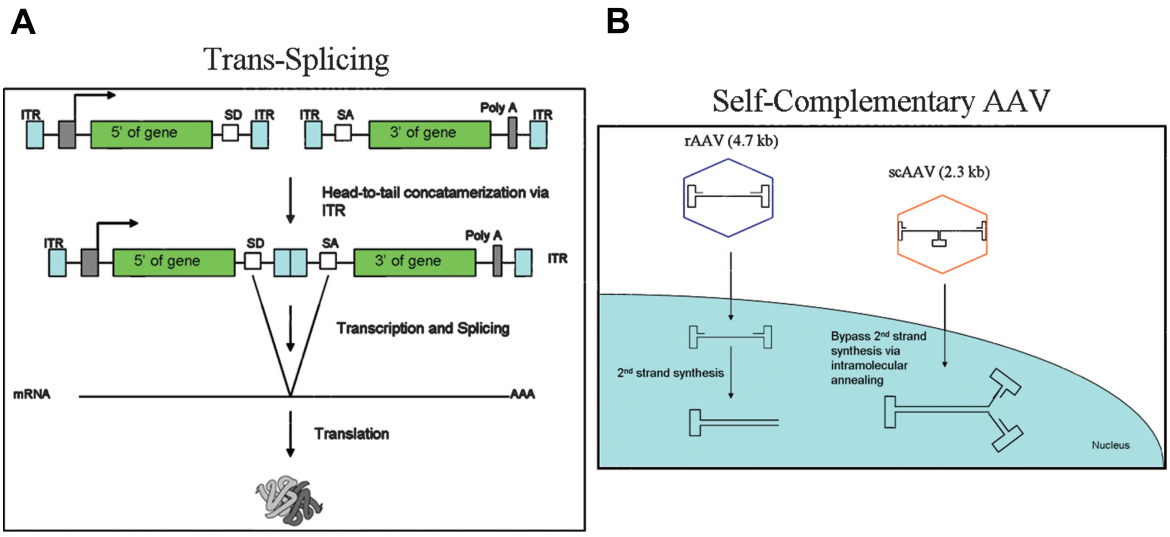


Figure 1.8 Different approaches for the production of AAV vectors.

A. Trans-splicing approach. The head-to-tail formation of two different AAV vector results in a functional product after splicing. **B.** Comparison of scAAV and rAAV vectors. The figure and legend are taken from Daya and Berns 2008 (34).

1.2.3 Immune Response Challenge

A major challenge to the success of AAV-based gene therapy is the high prevalence of pre-existing neutralizing antibodies (NA) due to prior infection with wild-type AAV (218, 144, 147), or the development of neutralizing antibodies upon readministration of the rAAV vector (129, 219, 169, 220). In fact, AAV immune response is mainly adaptive; almost no innate immunity is involved (221). AAV immune response implicates both humoral and cell-mediated immunity, though humoral immunity is chiefly involved (218, 222) and seems to be T-cell-dependent, as anti-CD4 antibodies prevented vector neutralization and allowed readministration of the vector with sustained expression, even after anti-CD4 level decreased (219). In most cases, pre-existing NA led to an impediment of transduction of the therapeutic gene (223-225). But AAV cell-mediated immune response plays an important role as well. AAV-specific T-cells were detected in healthy subjects and expanded *in vitro* upon AAV-mediated gene therapy in a dose-dependent manner (226, 227). Veron et al. showed that AAV1 specific T-cell response was correlated neither with the prevalence of anti-AAV antibodies nor with the prevalence of NA (228). Furthermore, CTL-mediated destruction of rAAV-transduced hepatocytes and an increase in transaminases was reported, and was associated with a concurrent decrease in the expression of factor IX in a clinical trial for haemophilia B (224). Several approaches have been developed to evade the immune response directed against rAAV vectors, including capsid pseudotyping (229, 230, 117), generation of mosaic capsids (231, 140), generation of a mutant library using error-prone PCR producing capsid with improved transduction efficiency even in the presence of antibodies (232-234), and brief immunosuppression (235, 236, 219, 223). However, it was recently shown that immunosuppression did not always result in improved AAV-mediated transduction or interference with AAV-specific immune response (237). The natural history of infection with AAV and the host immune interactions are not yet clear. This imposes further challenges and underlines the need for additional consideration of, and deeper insight into the AAV-human host interaction for better targeted and tolerated AAV vectors.

1.3 Aim of the Study

AAV is a promising gene therapy vector, however little is known about AAV *in vivo* biology. There is an inconsistency between the high AAV seroprevalence reported and the rather sporadic detection of AAV in human samples. The main focus of this study was to explore the target cells for AAV persistence in normal population. Based on previous studies and reports (please refer to section 1.1.9), we assumed that AAV might persist in the leukocytes. If AAV already persists in leukocytes of healthy donors, it might be reactivated as a consequence of reactivation of any coinfecting AAV helper viruses, which is common upon immunosuppression. Therefore, as we progressed in our study, the screening of AAV in immunosuppressed patients to clarify this postulation became our second goal.

2 Materials

2.1 Cell lines

Table 2.1 Cell lines

Cell Line	Description	Source	Reference
Detroit 6*	Human bone marrow continuous cell line - a clone from it latently infected with AAV2 was established by Bern and colleagues (67) and used in that study as a positive control.	-Laboratory stock -ATCC® CCL-3-discontinued -RRID: CVCL_2436	(238)
HEK293	Human embryonic kidney cell transformed with sheared adenovirus type 5 DNA.	-Laboratory stock -ATCC® No.CRL-1573™ -RRID: CVCL_0045	
HeLa*	Human immortal epithelial cell line derived from Henrietta Lacks cervical cancer cells.	-Laboratory stock -ATCC® No. CCL-2 -RRID: CVCL_0030	(239)
MRC-5*	Human embryonic fibroblast cells	-Laboratory stock. -ATCC® CCL-171™ -RRID: CVCL_0440	

* Chromosomal DNA extracted from HeLa and MRC-5 cell lines, and Detroit 6 cells served as negative and positive control templates in AAV sero PCR assay, respectively.

2.2 Bacteria

Table 2.2 Strains of bacteria

Clade	Genotype	Description	Source
E.coli SURE	F′[*proAB*$^{+}$ *lacI*^{q} *lacZΔM15* Tn*10* (Tet^{R}] *endA1 glnV44 thi-1 gyrA96 relA1 lac recB recJ sbcC u muC*::Tn*5*(Kan^{R} *uvrC e14*$^{-}$(mcrA^{-}) Δ*(mcrCB-hsdSMR-mrr)171*	For transformation by heat‐shock treatment	ATCC‐No.55695 Stratagene
XL1-Blue	endA1 gyrA96(nal^{R}) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB^{+} lacI^{q} Δ(lacZ)M15] hsdR17(r_K^- m_K^+)	For transformation by heat‐shock treatment	Stratagene

2.3 Plasmids

Table 2.3 Plasmids

Plasmid	Description	Source
pIR-VP- AAV1-hr2-RBE	Plasmid expressing AAV1 *cap* gene encoding structural proteins VP1, VP2, and VP3 with *hr* enhancer and RBE to up-regulate the P19 promoter, derived from previously described pIR-VP-hr2-RBE (240), where AAV2 *cap* has been replaced by AAV1 *cap* (204).	AG* Heilbronn
pTAV-2.0	Plasmid carrying the whole gene sequence of wild-type AAV2 (241).	AG* Heilbronn

Plasmid	Description	Source
pIR-VP-AAV3-hr2-RBE	Plasmid expressing AAV3 *cap* gene encoding VP1, VP2, and VP3, (same structure as pIR-VP- AAV1-hr2-RBE, but AAV2 *cap* has been replaced by AAV3 *cap*) (204).	AG* Heilbronn
pIR-VP-AAV5-hr2-RBE	Plasmid expressing AAV5 *cap* gene encoding VP1, VP2, and VP3, (same structure as pIR-VP- AAV1-hr2-RBE, but AAV2 *cap* has been replaced by AAV5 *cap*) (204).	AG* Heilbronn
pIR-VP-AAV6-hr2-RBE	Plasmid expressing AAV6 *cap* gene encoding structural proteins VP1, VP2, and VP3, (same structure as pIR-VP- AAV1-hr2-RBE, but AAV2 *cap* has been replaced by AAV6 *cap*) (204).	AG* Heilbronn
pIR-VP-AAV7-hr2-RBE	Plasmid expressing AAV7 *cap* gene encoding structural proteins VP1, VP2, and VP3, (same structure as pIR-VP- AAV1-hr2-RBE, but AAV2 *cap* has been replaced by AAV7 *cap*) (204).	AG* Heilbronn
pIR-VP- AAV8-hr2-RBE	Plasmid expressing AAV8 *cap* gene encoding structural proteins VP1, VP2, and VP3, (same structure as pIR-VP- AAV1-hr2-RBE, but AAV2 *cap* has been replaced by AAV8 *cap*) (204).	AG* Heilbronn

Plasmid	Description	Source
pIR-VP-AAV9-hr2-RBE	Plasmid expressing AAV9 *cap* gene encoding structural proteins VP1, VP2, and VP3, (same structure as pIR-VP- AAV1-hr2-RBE, but AAV2 *cap* has been replaced by AAV9 *cap*) (204).	AG* Heilbronn
prhAAV10	Plasmid expressing AAV10 *cap* gene encoding structural proteins VP1, VP2, and VP3, (same structure as pIR-VP- AAV1-hr2-RBE, but AAV2 *cap* has been replaced by rhAAV10 *cap*) (204).	AG* Heilbronn
pIR-VP-AAV11-hr2-RBE	Plasmid expressing AAV11 *cap* gene encoding structural proteins VP1, VP2, and VP3, (same structure as pIR-VP- AAV1-hr2-RBE, but AAV2 *cap* has been replaced by AAV11 *cap*) (204).	AG* Heilbronn
pIR-VP-AAV12-hr2-RBE	Plasmid expressing AAV12 *cap* gene encoding structural proteins VP1, VP2, and VP3, (same structure as pIR-VP- AAV1-hr2-RBE, but AAV2 *cap* has been replaced by AAV12 *cap*) (204).	AG* Heilbronn
pIR-VP-AAV13-hr2-RBE	Plasmid expressing AAV13 *cap* gene encoding structural proteins VP1,VP2, and VP3, (same structure as pIR-VP-AAV1-hr2-RBE, but AAV2 *cap* has been replaced by AAV13 *cap*) (204).	AG* Heilbronn

*AG stands for Arbeitsgruppe, the German synonym for the working team.

2.4 Antibodies

Table 2.4 Antibodies used for MACS sorting

Antibody	Description	Source
Human CD3 MicroBeads	MicroBeads conjugated to monoclonal anti-human CD3 antibodies (isotype: mouse IgG2a).	Miltenyi Biotec, Germany
Human CD14 MicroBeads	MicroBeads conjugated to monoclonal anti-human CD14 antibodies (isotype: mouse IgG2a).	Miltenyi Biotec, Germany

2.5 Oligonucleotide Library

All oligonucleotides were provided by Eurofins MWG Operon GmbH in lyophilized form brought to the standard concentration for stock solutions of 100 pmol/µl by dissolving with the appropriate diluents' volume indicated by the manufacturer. The working solution concentration was 10 pmol/µl. The diluent used was DNase, RNase, Protease, free water (Sigma-Aldrich). Both stock and working solutions were stored at -20 °C.

2.5.1 Oligonucleotides Used as Primers in the First Round PCR (Sero PCR)

Oligonucleotides	Sequence (5′→3′)
AAVsero for	5′-TAC AAG TAC CTC GGA CCC TTC AAC-3′
AAVsero rev	5′-TGG AAT CGC AAT GCC AAT TTC C-3′
AAV5 for	5′-GCT GCC TGG TTA TAA CTA TCT CGG-3′

2.5.2 Oligonucleotides Used as Primers in Sero Nested PCR

Oligonucleotides	Sequence (5´→3´)
AAVsero nest for	5′-AGA TAC GTC TTT TGG GGG CAA C-3′
AAVsero nest rev	5′-CGT TAT TGT CTG CCA TTG GTG C-3′

2.5.3 Oligonucleotides Used as Primers in AAV5 Nested PCR

Oligonucleotides	Sequence (5´→3´)
AAV5 nest for	5′-TCA AGT ACA ACC ACG CGG AC-3′
AAV5 nest rev	5′-ACT CCA TCG GCA CCT TGG TTA-3′

2.5.4 Oligonucleotides Used as Primers in Cap1 PCR

Oligonucleotides	Sequence (5´→3´)
Cap1 for	5`-GAC AGG TAC CAA AAC AAA TGT TCT C-3`
Cap1 rev	5`-GGA ATC GCA ATG CCA ATT TCC-3`

2.5.5 Oligonucleotides Used as Primers in Cap2 PCR

Oligonucleotides	Sequence (5´→3´)
Cap2 for	5`-ACC AAT GGC AGA CAA TAA CG-3`
Cap2 rev	cap2 rev: 5`-CCA AAG TTC AAC TGA AAC GAA-3`

2.5.6 Oligonucleotides Used as Primers in Cap2 PCR

Oligonucleotides	Sequence (5´→3´)
Cap2_new for	5`-GCA CCA ATG GCA GAC AAT AAC G-3`
Cap2_new rev	5`-GCT GTT TTC CTT CTG CAG CTC-3`

2.6 Nucleotides

dNTPs (dATP, dTTP, dCTP, dGTP)	Fermentas, NEB

2.7 Standard Marker

GeneRuler^{TM} DNA Ladder Mix	Fermentas, Thermo Fischer Scientific

2.8 Enzymes

Benzonase	Merck
Herculase (Proofreading hot-start polymerase)	Agilent Technologies
Platinum Polymerase	Thermo Fischer Scientific
Proteinase K	Roth
Q5 High-Fidelity DNA Polymerase	New England Biolabs
Restriction buffer; NEB3.1, NEB4, CutSmart	New England Biolabs
Restriction enzymes Rsa-I, Swa-I Xba-I, Xho-I .	New England Biolabs
RNase A	Genomed
Taq DNA Polymerase, with ThermoPol buffer	New England Biolabs

2.9 Kits

CloneJET PCR Cloning Kit	Thermo Fischer Scientific
DNeasy Blood & Tissue Kit	Qiagen
GeneMATRIX 3 in 1- Basic DNA Purification Kits	EURx
GeneMATRIX Quick Blood DNA Purification Kits	EURx
JetStar™ 2.0 Plasmid Purification Kit	Genomed
LightCycler Kit- Fast Start DNA Master SYBR Green I	Roche
QIAamp DNA Blood Mini Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen, Hilden, Germany
LS columns	Miltenyi Biotec, Germany

2.10 Culture Media and Additives

DMEM high glucose (Gibco®)		
Additives:	10%	Fetal calf serum
	100µg/ml	Penicillin
	100µg/ml	Streptomycin
LB Medium	1% (w/v)	Bacto Tryptone
	0.5% (w/v)	Bacto Yeast extract
	1% (w/v)	NaCl
	for LB plates:	
	1.5% (w/v)	Bacto Agar

LB-Medium with Ampicillin		
	100 µg/ml	Ampicillin in LB medium
LB-Plates with Ampicillin	1,5% [w/v]	Agar, LB-Medium
	100 µg/ml	Ampicillin
RPMI 1640 Medium (Gibco®)		
Additives:	100µg/ml	Penicillin
	100µg/ml	Streptomycin

2.11 Buffers/Reagents

ACK lysis buffer		GE
DNA loading buffer (10x)	50 mM	EDTA pH 8.0
	30 %	Ficoll 400
	2 %	SDS
	0,25 %	Bromophenol‑blue
	0,25 %	Xylene cyanol
DNA-Lysis buffer (3x)	3 %	N-Lauryl Sarcosylate
	70 mM	Tris, pH 8,5
	25 mM	EDTA pH 8,0
MACS Buffer	1x	PBS
	2 mM	EDTA
		Buffer was sterilized by autoclaving, kept at 4ºC.

PBS (1x)	18.4 mM	Na_2HPO_4
	10,9 mM	KH_2PO_4
	125 mM	NaCl, pH7.2
STET Buffer	80 g/l	Saccharose
	5 % [v/v]	Triton X-100
	50 mM	EDTA, pH 8,0
	10 mM	Tris/HCl, pH 8,0
TAE (1x)	40 mM	Tris‐Acetate
	1 mM	EDTA, pH 8.0
		With Acetic Acid 100 %, pH set to 8.5
TE (1x)	10 mM	Tris‐HCl pH 8.0
	1 mM	EDTA

2.12 Chemicals

Reagent	**Company**
2-Propanol	Roth
Agar	Becton, Dickinson and Company
Bacto™ Peptone	Becton, Dickinson and Company
Bacto™ Trypton	Becton, Dickinson and Company
Bovine serum albumin (BSA)	GE Healthcare
D(+)-Saccharose	Roth
Deoxyribonucleic acid sodium salt from salmon testes	Sigma –Aldrich
Ethanol	Roth

Ethidium bromide 1% (10 mg/ml)	Roth
Ethylene diamine tetra acetate (EDTA)	Roth
Fetal calf serum (FCS)	Gibco®
Glycerine (86 %)	Roth
Midori Green	Nippon Genetics
Natrium acetate	Merk
Penicillin/Streptomycin	Biochrom
Roti- Phenol- Chloroform- Isoamyl alcohol (25/24/1)	Roth
Salmon testes DNA	Sigma
Sodium chloride	Roth/ Merk
Sodium dodecyl sulphate (SDS), ultra pure	Roth
Tris (Tris (hydroxymethyl)-aminomethane)	Roth
Triton X-100	Merk
Trypsin-EDTA (0. 5 (10x)	Gibco®

2.13 Solutions

Agarose solution	0.8-2 % [w/v]	Agarose in 1×TAE buffer
Ampicillin-Stock solution	100 mg/ml	Ampicillin in dd. H_2O
Lysozyme	10 mg/ml	Lysozyme in dd. H_2O
Sodium acetate	3M	Na-Acetate in ddH_2O, pH 7.0

2.14 Consumables

Cell culture dishes	Ø 3/6/10/15 cm	Sarstedt
Cell culture flasks	75/175 cm^2	Sarstedt
Cell culture plates	6/12/24/48/96 well	Sarstedt
Cell scraper		Costar
Cover slides	Ø 1.5 cm	Langenbrinck
Nunc® CryoTube®	1.8 ml	Sigma –Aldrich
Eppendorf tubes	0.5/1/2 ml	Eppendorf
Falcon tubes	15/50 ml	BectonDickinson (BD)
Rotilabo®- Syringe filter	0.22 µm	Roth
Filter paper	Ø 125 mm	Schleicher & Schuell
Needles	0.8x120 mm	B Braun Sterican®
Counting chamber		HBG, Neubauer
Parafilm M®		Bemis
Pasteur pipette	230 mm	WU Mainz
PCR-Reaction tubes strip		Applied Biosystems
PCR-Reaction tubes with attached cap		ThermoFisher Scientific
Petri dishes	10 cm	Greiner
Pipette tips	2/20/100/200/1000 µl	Sarstedt
Reaction tubes	0,5 ml, 1,5 ml, 2 ml	Sarstedt
Syringes	3 cm, 10 cm, 50 cm	BD

2.15 Equipment

Agarose gel electrophoresis apparatus	-VWR 300v -Biorad Power PAC 300
Avanti J-25 centrifuge (Rotor JLA 16250)	Beckmann
Avanti JE centrifuge	Coulter, Beckmann
Benchtop microcentrifuge, 5415D (rotor F 45-24-11)	Eppendorf
Electronic precision scales	Sartorius
Freezer (-20ºC, and -80ºC)	AEG/Bosch/GFL/Liebherr
Gel documentation system UV-system	Intas
Incubation shaker	-Infors (AG) HT -New Brunswick Scientific
Incubators: HeRa cell 240 HeRa cell	-Heraeus -Thermo Scientific
Inverse light microscope	Olympus
Isopropanol bath	-
Laminar air flow cabinets with UV	-BDK -Gelaire flow laboratory, BSB 4.4
Liquid nitrogen tank	Isotherm
Magnetic stirrer	Schott (Duran)
Measuring cylinders	Eterna, Brand W-Germany
Megafuge 3.0R	Heraeus Sepatech
Megafuge 3.0R centrifuge (rotor 8074)	Heraeus SEPTACH
MidiMACS™ separator	Miltenyi Biotec, Germany
Mikro 22R centrifuge	Hettich
MyCycler™ thermocycler	Biorad
Overhead shaker	Heidolph
pH- Meter	Mettler Toledo

Pico 17 microcentrifuge (24-Pl Rotor)	Thermo Scientific Heraeus
Pipetboy	-IBS Integra Bioscience -Brand
Refrigerator	Liebherr
Spectrophotometer	Beckman Du530
Thermal cycler	-Applied Biosystems -Bio-Rad
Thermomixer	Eppendorf
UV-transilluminator	Laborgeräte Vetter GmbH
Vacuum centrifuge DNA SpeedVac DNA 110	Savant
Vortex mixer	Scientific Industries
Water bath	-Julapo PC - Fisher Scientific

2.16 Computer software

Adobe Illustrator
Adobe Photoshop CS5.1
Codon Code Aligner (CCA)
Endnote X7
Gene Construction Kit™ 3.5
ImageJ2 (242, 243)
MEGA6.06 (244)
Microsoft Excel 2011
Microsoft Word 2011
Splitstree4 (245)
Spss22

2.17 Data Bank Resources

Multiple sequence alignment program (ClustalW2) (246)

Multiple Alignment using Fast Fourier Transform (MAFTT) (247)

The Restriction Enzyme Database (REBASE) (248)

Chromosome sequences from International Human Genome Project, hg18 (249)

Ensemble (250)

GeneCards

NCBI CDD (251)

NCBI (252)

UniProt (253)

3 Methods

3.1 Cell Biology Methods

3.1.1 Cell Culture

Adherent cell lines were cultivated in monolayer cultures in DMEM. Cells were incubated in cell culture plates/flasks at 37°C, 5 % CO_2 and 95 % humidity. For cell propagation, the medium was aspirated and cells were washed with PBS, then 1-5 ml 0.05 % trypsin was added and incubated at 37°C for 2-4 min. As soon as the cells were detached, they were resuspended in fresh medium to deactivate the trypsin. Cells were further cultivated according to the dilution factor (1:2 to 1:20) or the intended cell number for the next experiment. To count the cells, 10 µl cell suspension was transferred to a haemocytometer and cells were counted on either counting grid. The mean value of the cell number in the four big squares was calculated. Cell count per ml equals the mean value of cell number multiplied by the dilution factor and chamber factor (1×10^4).

3.1.2 Cell Cryopreservation

Cryopreservation is necessary to store cell line stock for further subculture; however, this can only be done for cells with high viability that withstand the stress caused during the procedure. After withdrawing the medium, cell monolayer was washed twice with PBS, briefly incubated with trypsin and resuspended in medium. Cells were pelleted by centrifugation (5 min, 900 rpm). The supernatant was discarded and the cell pellet resuspended in 1.5 ml cryopreservation medium (DMEM, with 20% FCS, and 10% DMSO) in cryovials (1.8 ml), which were then placed in an isopropanol bath and allowed to cool down slowly to -80°C in a freezer overnight (O/N), then they were transferred to a liquid nitrogen tank. For further use, the cryovials were thawed in a water bath; then the medium was added quickly to minimize the cryoprotectant's (i.e. DMSO) toxic effect (254).

3.1.3 Density- Gradient Separation of Peripheral Blood Mononuclear Cells Using Ficoll.

To further identify the target cell subtype for AAV persistence, peripheral blood mononuclear cells (PBMCs) were obtained from leukocytes and further separated into different fractions. Freshly withdrawn-EDTA blood (50 ml) was diluted with sterile PBS 1:2, then 16 ml of

Ficoll-Paque Plus (GE Healthcare) was added in new conical 50 ml Falcon® tubes, and overlaid with 32 ml diluted blood, which was added very slowly and carefully on the wall of the tube and not directly over the Ficoll layer to avoid mixing up the two layers. The tubes were carefully placed in a swinging bucket centrifuge for centrifugation (800 *x g*, RT, 35 min) without brake. The upper plasma layer was carefully withdrawn without disturbing the underlying PBMCs layer, then the grey ring (buffy coat) was transferred to a new 50 ml tube (up to 3 rings/tube). The tube was filled with PBS to ~50 ml and centrifuged at 300 *x g*, 10 min at 4°C. To lyse contaminating RBCs, the pellet was resuspended in 5 ml ACK-lysis buffer (Life Technologies) and incubated 5 min, then filled to 20 ml with PBS and spun at 200 *x g*, 10 min at 4°C. The pellet was resuspended in 20 ml PBS, centrifuged at 100 *x g*, 10 min at 4°C. The total cell number was determined by resuspending the pellet in 10 ml PBS and counting the cell number in about 10 µl on a Neubauer - counting chamber. The cells were then pelleted again at 300 *x g*, 10 min at RT.

3.1.4 MACS Separation of $CD14^+$ or $CD3^+$ Cells

Magnetic-activated cell sorting (MACS) allows the separation of cells expressing certain antigen on their surface. Magnetic microbeads bind to the cellular antigen of interest, and allow this cell fraction to stay on the column attached to the MACS adaptor due to magnetic field, while unbound cells pass through. Target cells bound to the magnetic microbeads were then eluted after removing the column away from the magnetic field. ($CD3^+$, and $CD3^-$) cells, or ($CD14^+$, $CD3^+$, and $CD14^-/CD3^-$) cells were isolated by two successive rounds of immunomagnetic bead selection using (CD3 microbeads), or (CD14 followed by CD3 microbeads), respectively, as well as LS columns (Miltenyi Biotec).

The cell pellet (from the last step in 3.1.3) was resuspended in 80 µl MACS buffer / 10^7 cells, then 20 µl $CD14^+$ microbeads / 10^7 cells were added and gently mixed and incubated for 20 min at 4°C. The cap was kept loose to allow the cells to aerate. Then, 1-2 ml PBS was added, and the cells were centrifuged at 300 *x g*, 10 min at 4°C. Meanwhile, the MACS separator was removed from the refrigerator, and the column was put into position (LS, Miltenyl) and washed with 3 ml MACS buffer. The cell pellet resuspended in 500 µl MACS buffer/10^8 cells was loaded onto the column. The flow-through (depleted cell fraction) was collected. The column was then washed three times with 3 ml buffer. The column was removed from the MACS adaptor and placed away from the magnetic field in a new collection tube, and after the addition of 5 ml MACS buffer, the plunger was applied and

pressed to collect the selected cell fraction ($CD14^+$ cells). The flow-through was reapplied to the column and recollected to increase the purity of the depleted cell fraction ($CD14^-$). The $CD14^-$ cells were then centrifuged (300 *x g*, 10 min, RT), and further separated into $CD3^+$ and $CD14^-/CD3^-$ cells using CD3 microbeads.

To separate $CD3^-$ and $CD3^+$ fractions, the same steps were repeated as in 3.1.4 but using CD3 microbeads instead.

3.2 Molecular Biology

3.2.1 Transformation of $CaCl_2$ Competent Bacteria

Competent cells (E.coli strains SURE and XL1 Blue) preserved in 0,1 M CaCl2/14% glycerine, were left to thaw slowly on ice. Fifty microliters of competent cells was mixed with 1 ng-1 µg plasmid DNA or 10 µl of ligation mixture and cooled on ice for 10-20 min. Cells were exposed to heat shock at 42ºC for 90 s, then cooled on ice for 2 min. 150 µl of pre-warmed LB medium was added, and the mixture was incubated for 1-2 h at 37ºC with gentle agitation (150-200 rpm). For cloning, different amounts of the mixture were plated out on LB agar plates with the appropriate antibiotic (end concentration 100 µg/ml) to select only transformed bacterial colonies with the plasmid carrying the antibiotic resistance. For midi and maxi plasmid preparation, the whole transformation mixture was added to 250 µl LB medium containing antibiotic. Either the plates or the medium tubes were incubated 12-16 h at 37ºC.

3.2.2 Plasmid DNA Preparation on a Small Scale (Minipreparation)

Plasmid DNA preparation from bacteria was done using the alkaline lysis method (255). The aim was to obtain a small amount of plasmid DNA sufficient to analyse bacterial clones by restriction digestion analysis to confirm ligation, or to submit plasmid DNA for sequencing.

A single colony was selected and inoculated into 3 ml LB medium with a selective antibiotic for 12-16 h at 37°C with continuous gentle shaking. One ml of overnight bacterial culture was centrifuged (14000 rpm, 4°C, 5 min) and after the medium was thoroughly withdrawn, the cell pellet was resuspended in 150 µl STET buffer. Thereafter, 12 µl of freshly dissolved lysozyme (10 mg/ml) was added, and all reagents were mixed well by short vortexing and incubated at 90°C for 60 s. Cell debris was pelleted by centrifugation (14000 rpm, 10 min, 4°C) and removed using a pipette tip. Plasmid DNA was precipitated with ethanol (see

3.2.7.3). The DNA pellet was allowed to dry (glass-like) and then dissolved in 30-50 µl TE, and kept at -20°C. For further processing of the clones, 100 µl of the corresponding overnight culture was inoculated in 100, or 250 ml LB medium for midi or maxi-preparation, respectively.

3.2.3 Plasmid DNA Preparation on a Large Scale (Midi-/Maxipreparation)

For the production of a larger amount of plasmid, midi- or maxi- plasmid preparations were carried out, and plasmid DNA was purified employing anion-exchange chromatography. JetStar™ 2.0 Plasmid Purification Kit was used to extract plasmid DNA according to the manufacturer's instructions. Briefly, cells were pelleted by centrifugation at 5000 rpm, 5 min, 4°C, then resuspended in 4/10 ml E1 buffer with RNase (midi/maxi) and incubated at RT 5 min after addition of buffer E2. Thereafter, 4/10 ml of buffer E3 was added and mixed, and the mixture was centrifuged at 10000 rpm, 5 min, 4°C, and allowed to pass through filter paper to an equilibrated anion exchange column (with 5/15 ml E4 buffer). The columns were then washed using 2x (10/30 ml) E5 buffer to remove genomic nucleic acids. The supercoiled plasmid DNA bound to the column's membrane was eluted in 5/15 ml E6 buffer, and then concentrated by ethanol precipitation (see 3.2.7.3). DNA concentration was determined using a spectrophotometer (see 3.2.7.6).

3.2.4 Restriction Digestion Analysis

Restriction enzymes are unique enzymes that recognize certain short DNA sequences (mostly 4-8 bases) and cleave DNA within or adjacent to these recognition sites. Restriction digestion can be used to analyse plasmid or to prepare DNA (plasmid or PCR fragments) for ligation and cloning.

The recommended buffer, reaction temperature, and addition of BSA, were determined according to the manufacturer's instructions, and the reaction was carried out using 4 µg DNA, and 10-20 units of the restriction enzyme at a total volume of 20 µl, and incubated at the recommended temperature for 2 h or O/N. To separate the DNA fragments according to their corresponding molecular weight, the restriction mixture was loaded on agarose gel (see 3.2.5).

3.2.5 Agarose-Gel Electrophoresis

To separate the DNA fragments according to fragment sizes, agarose gel electrophoresis was performed. Agarose was heated in 1x TAE till it completely dissolved, then allowed to cool down to 60°C. Thereafter, the DNA intercalating dye Midori Green Advanced DNA Stain (1µl / 20ml) was added, mixed well and poured into a gel chamber with an inserted gel comb. When the gel hardened, the casting gates and gel comb were removed, and the gel was overlaid with TAE buffer (1 mm). Alternatively, no DNA intercalating dye was added, and after DNA fragments had migrated onto the gel, the gel was placed in an ethidium bromide bath (0.5 µl/ 1ml) for 30 min, then destained in a water bath for 10-30 min. The agarose concentration (1% -2% (w/v)) depended on the size of the DNA fragments to be separated, and the current used depended on the size of the gel and ranged from 80 to 130 volts (v). The gel was observed under a UV transilluminator and recorded photographically.

3.2.6 Isolation and Purification of DNA Fragments from Agarose Gel

To isolate specific DNA fragments from agarose gels, DNA fragments were visualized with long wavelength UV light (366 nm), and the gel slice with the desired DNA fragments was cut out as fast as possible to minimize UV damage to the DNA. The excised DNA fragments were purified using GeneMatrix 3 in 1- Basic DNA purification kits according to the manufacturer's instructions. The DNA concentration was estimated by reference to the indicated size of the corresponding band on the molecular weight marker.

3.2.7 Genomic DNA Extraction

Genomic DNA was extracted using spin column kits according to the manufacturer's instructions or by phenol-chloroform extraction.

3.2.7.1 Cell Lysis for Genomic DNA Extraction from Culture Cells

When cells reached confluency, the medium was aspirated, and cells were washed with 2x PBS and detached with trypsin. When infected cells showed CPE, they were harvested by cautious withdrawal of medium and scraped to collect undetached cells as well. The cells were pelleted (1000 rpm, 10 s), washed with PBS, and pelleted again. The cell pellet was resuspended in 250-300 µl 10 mM Tris (pH 8,5), or PBS or medium, to which 150 µl 3x DNA lysis buffer was added and mixed well; then 5 µl proteinase k (10 mg/ml) was added, and the mixture was incubated at 56°C, 2 h. The role of proteinase k is to deactivate nuclease and to digest any

other DNA-bound protein, rendering extracted DNA pure and intact. If the DNA extracted was intended for further enzymatic reaction with which the co-presence of RNA might interfere, an RNA digestion was carried out by adding 20 µl RNase A (10 mg/ml) prior to the addition of lysis buffer.

3.2.7.2 Phenol-Chloroform Extraction

Genomic DNA from HeLa cells was purified using phenol-chloroform extraction. The principle is to isolate DNA in the upper aqueous phase, whereas cell debris and proteins are being retained in the organic layer. Phenol, phenol/chloroform (24/1), and phenol/chloroform/isoamyl alcohol (25/24/1) were added sequentially (1:1 v/v) and mixed thoroughly with the sample by inverting the tubes several times for 8 min or by vigorous vortexing for 10 s. The mixture was centrifuged at 13,000 rpm for 8 min. The aqueous upper phase containing the DNA was carefully transferred to a new microcentrifuge tube and precipitated with 2.5 volume of ethanol (see 3.2.7.3).

3.2.7.3 Ethanol/ Isopropanol DNA Precipitation

To remove any traces of phenol and to precipitate pure DNA, the upper aqueous phase was added to a new reaction tube containing (1/10 v/v) 3 M sodium acetate (pH 5,2), briefly mixed by vortexing, followed by the addition of 2.5 vol. of ice-cold 100 % ethanol, and thorough mixing, and short incubation at 4°C, then centrifugation at 13,000 rpm for 10 min, 4°C. Notably, storage of the sample at 4°C or -20°C O/N before centrifugation markedly increased the DNA yield. The supernatant was aspirated, and the DNA pellet was washed with room-temperature 70 % ethanol and centrifuged again. The DNA pellet was air- or vacuum- dried and dissolved in 30-150 µl TE, pH 7.4. The DNA concentration was determined using a spectrophotometer (see 3.2.7.6).

3.2.7.4 Spin Column Kit

Alternative to phenol-chloroform extraction, spin column kits were used to extract DNA either from culture cells, i.e. Detroit 6 or HeLa cells using DNeasy Blood & Tissue Kit (Qiagen) or from leukocytes. For DNA isolation from human blood samples, EDTA blood samples (5-10 ml) collected for routine diagnostics were centrifuged at 4000 x *g* at room temperature for 5 min. DNA was extracted from 200-400 µl of buffy coat, using QIAamp DNA Blood Mini Kit (Qiagen). Genomic DNA from HeLa cells, which served as negative control in PCR, was extracted using QIAamp DNA Blood Mini Kit along with blood samples

to ensure purity of all reagents and exclude contamination.

To concentrate the DNA extracted from immunosuppressed patients' blood samples after isolation using the spin kits, ethanol precipitation was performed (see 3.2.7.3), and the DNA pellet was dissolved in 30 µl Tris, pH 7.4.

3.2.7.5 DNA Extraction From Different Leukocytes' Fractions

Different purified leukocyte fractions, i.e. $CD14^+$, $CD3^+$, $CD14^-$, were centrifuged (4000 *x g*, 10 min, RT), the pellet was resuspended in 200 µl PBS and DNA was extracted using QIAamp DNA Blood Mini Kit (Qiagen) as described above (see 3.2.7.4).

Extracted DNA was analysed by AAV nested PCR assay as described (3.2.8.1). AAV copy numbers were quantified by real-time PCR. Samples and standards were preamplified by 20 cycles AAV 1st round PCR. A real-time LightCycler PCR followed, using 2.5 µl of 1st round PCR product as a template, 500 nM AAVsero nest primers and GoTaq qPCR Master Mix (Promega), containing GoTaq® Hot Start Polymerase, $MgCl_2$, dNTPs, BRYT Green® dye and the reaction buffer, at a final volume of 10 µl. Amplification conditions were: 95°C for 15 min, followed by 40 cycles at 95°C for 15 sec, 60°C for 20 sec and 72°C for 30 sec.

3.2.7.6 Determination of the DNA Concentration

DNA concentration was determined using optical density (OD) measurement. Determination of DNA concentration is a very crucial tool to standardize the amount of template used as input DNA in PCR or restriction digestion. Also, the ratio between the absorbance of DNA and protein gives an indication of the purity of the DNA. DNA absorbs light at OD_{260nm}, while protein absorbs light at OD_{280nm}. Using a quartz cuvette, and a spectrophotometer with a UV lamp, DNA concentration can be deduced with the following equation:

DNA concentration (µg/ml)= OD_{260} x dilution factor x multiplication factor.

Multiplication factor for dsDNA=50, and for ssDNA=33.

3.2.8 Polymerase Chain Reaction (PCR)

PCR is a milestone in molecular biology. It was first described in 1985 by Saiki and colleagues (256), while originally developed by Mullis and Faloona (257). It consists of three main steps occurring in consecutive cycles: first, denaturation of the double-stranded DNA,

followed by annealing of specific oligonucleotides primers that are complementary to the template, now single-stranded, DNA. Then an extension step follows, where the heat-stable polymerase continues adding new nucleotides until a new copy of the original DNA template forms. At the end of each cycle, the number of DNA templates doubles. Theoretically, beginning with one copy of the template should yield 1 million copies after 32 cycles under optimal conditions. It sounds like a simple technique, but it is rather tricky to optimize and its troubleshooting can be sometimes very time-consuming.

3.2.8.1 Establishment of Nested AAV PCR Assay

3.2.8.1.1 Establishment of First Round Sero PCR

Up to 1 µl genomic DNA was used as a template in PCR. The PCR reaction mixture consisted of the primers AAVsero for, AAVsero rev, and AAV5 for (0.6 µM each), 0.22 mM dNTPs, and 5 U of proofreading hot-start polymerase (Herculase, Agilent Technologies) in reaction buffer at a final volume of 50 µl (Table 3.1).

Table 3.1 Composition of first round PCR reaction mixture

Components	**Volume**
DNA (1 µg)	It varied; based on photometric estimation of DNA concentration
10 x reaction buffer	5 µl
AAVsero for (10 pmol/µl)	3 µl
AAVsero rev (10 pmol/µl)	3 µl
AAV5 for (10 pmol/µl)	3 µl
dNTPs (10 mM)	1.14 µl
Herculase (2,5 U/µl)	1 µl
DNase, RNase-free water	Add to final volume 50 µl
Total volume	50 µl

Thermocycling profiles of PCR consisted of an initial denaturation step at 94°C for 2 min, followed by 45 cycles of 94°C for 20 sec, 61°C for 30 sec, and 72°C for 1 min, then a final extension step at 72°C for 7 min. Non-template (nuclease-free water) and total genomic DNAs from 10^7 HeLa cells were used as negative controls to ensure purity of all reagents used for DNA extraction and PCR mixture.

AAV plasmids carrying the *cap* gene of AVV1-3 and 5-13 (pIR-VP- AAV1-hr2-RBE (204), pTAV 2-0 (241), pIR-VP- AAV3-hr2-RBE, pIR-VP- AAV5-hr2-RBE, pIR-VP- AAV6-hr2-RBE, pIR-VP- AAV7-hr2-RBE, and pIR-VP- AAV8-hr2-RBE, pIR-VP- AAV9-hr2-RBE, prhAAV10, pIR-VP- AAV11-hr2-RBE, pIR-VP- AAV12-hr2-RBE, pIR-VP- AAV13-hr2-RBE (204)), were linearized using Xba-I (AAV1-3, 5-8, 10-12) or Swa-I (AAV9, 13) and column-purified (QIAquick PCR Purification Kit, Qiagen). Serial dilutions (from $2e^8$ to $2e^0$ copies / µl) were prepared in H_2O or on the background of genomic DNA to determine PCR sensitivity and simulate the conditions in human samples.

3.2.8.1.2 Establishment of Nested Sero PCR

For higher sensitivity and to eliminate unspecific products, a dynamic touchdown PCR was adapted. The PCR program is summarized in Table 3.2.

In our protocol, the strategy in the first 18 cycles was to give an advantage to the specific template and exclude any unspecific product. In the next 17 cycles, due to consumption of dNTPs, primers and the exhaustion of Herculase, the stringency of PCR conditions was lowered for more efficient amplification of the specific product, which should already outcompete any unspecific amplicon.

Table 3.2 Thermocycling profile of touchdown nested PCR

Step	No. of cycles	Duration	Temperature
Initial denaturation	1×	2 min	94°C
Denaturation Annealing Extension	8×	20 sec 30 sec 40 sec	94°C 63.5°C (↓ 0.5°C/cycle) * 72°C
Denaturation Annealing Extension	10×	20 sec 30 sec 40 sec	94°C 60°C 72°C
Denaturation Annealing Extension	10×	20 sec 30 sec 50 sec	94°C 60°C (↓ 0.5°C/cycle) 72°C
Denaturation Annealing Extension	7×	20 sec 30 sec 50 sec	94°C 55°C ** 72°C
Final elongation	1×	7 min	72°C
Cooling	1×	∞	4°C

* For AAV5 nested PCR, the annealing temperature at that step is 64°C, ** and at that step 57°C.

The nested PCR mixture was composed of 0.2 mM dNTPs, AAV nest forward primer and AAV nest reverse primer (0.5 µM each) in reaction buffer, and 2.5 U proofreading hot-start polymerase (Herculase, Agilent Technologies). For positive samples as well as positive controls, 1 µl of 10^{-2} diluted 1st round PCR product was used as template for nested PCR,

while for samples that did not show any signal in the 1st round PCR as well as negative controls, 1 µl was used as template. In addition, 1 µl new non-template negative control was tested to exclude contamination in either PCR round (Table 3.3).

Table 3.3 Composition of nested sero PCR reaction mixture

Components	Volume
Template	(1 µl of 1st round PCR product or 1 µl of new non-template negative control)
10 x reaction buffer	5 µl
AAVsero nest for * (10 pmol/µl)	2.5 µl
AAVsero nest rev * (10 pmol/µl)	2.5 µl
dNTPs (10 mM)	1 µl
Herculase (5 U/µl)	0.5 µl
DNase, RNase-free water	37.5 µl
Total volume	50 µl

* The same reaction mixture composition was used for AAV5 nested PCR but using AAV5nested primer pair instead of the AAVsero nest primers.

To avoid sample-to-sample contamination and from co-workers′ samples, DNA extraction was carried out in a laminar flow cabinet with a UV-light hood treated with UV light overnight before and after sample processing in a specific room. Besides, separate rooms and dedicated equipment were used for the PCR setup. The PCR setup hood was also treated with UV light overnight before setting up any PCR amplification. Filter tips were always used. Non-template and cellular genomic DNA negative controls were always tested along with experimental samples.

3.2.8.1.3 Establishment of AAV5 Nested Sero PCR

As mentioned before, AAV5 is the most divergent serotype, so a separate nested PCR for AAV5 had to be developed. A separate nested AAV5 primer pair was generated (AAV5 nest for (*nt* 2475-2494) and AAV5 nest rev (*nt* 2818-2838)), leading to an amplicon of 364 bp. The reaction mixture was the same as for AAV sero nested PCR (Table 3.3). The thermal cycling conditions were also the same, except for the annealing temperature of 64°C for the first eight cycles, and the steady annealing temperature of 57°C in the last seven cycles (Table 3.2).

3.2.8.2 Establishment of Cap1/Cap2 PCR

Cap PCR was developed to amplify the capsid-coding region from selected AAV-positive samples, where the sequenced isolate has ≤ 98% homology with known human AAV serotypes. Due to the length of the *cap* gene, two primer pairs were generated, cap1 and cap2 primers, amplifying the 5` half of the *cap* gene as a 1-kb PCR product, while the 3` half was amplified as a 1.4 - 1.6-kb amplicon. Two different primer pairs, cap2 and cap2_new, in cap2 PCR, were alternatively used to amplify the 3` half, depending on which one amplified PCR fragments more efficiently. The PCR reaction for both cap1 and cap2 PCR was composed of 0.2 mM dNTPs, cap for and cap rev primers (0.5 µM, each), 1x reaction buffer, and 2-4 U proofreading hot-start polymerase (Herculase) supplied by Agilent Technologies at a final volume of 50 µl. Thermocycling profiles for cap1 PCR consisted of initial denaturation at 94°C for 2 min and 40 cycles of 94°C for 20 sec, 60°C for 30 sec, and 72°C for 70 sec, followed by a final extension step at 72° C for 7 min. The PCR conditions employed for cap2 PCR were one cycle of 94°C for 2 min and 40 cycles of 94°C for 20 sec, 58°C for 30 sec, and 72°C for 110 sec followed by a final extension step at 72°C for 7 min. PCR conditions with cap2_new primers were as follows: 1 cycle of 94°C for 2 min and 45 cycles of 94°C for 20 sec, 59°C for 30 sec, and 72°C for 130 sec, followed by a final extension step at 72°C for 7 min (Table 3.4). Non-template (nuclease-free water) and total genomic DNA from HeLa cells were used as negative controls, while pTAV-2.0 was used as positive control and to estimate the sensitivity of PCR. The cap1 primers match serotypes AAV-1- 3, 6, 7, 8 and 10 completely, while there are two mismatches for AAV11 and 12 in the reverse primer. Cap2 primers match five serotypes: AAV-1-3 and 6-8.

Table 3.4 Summary of PCR conditions of cap PCR

Step	Cap1 PCR	Cap2 PCR	Cap2_new PCR
Initial denaturation	1× 94°C, 2 min		
$\times$ {Denaturation / Annealing / Extension	$\times 40$ {94° C, 20 sec / 60° C, 30 sec / 72° C, 70 sec	$\times 40$ {94° C, 20 sec / 58° C, 30 sec / 72° C, 110 sec	$\times 45$ {94° C, 20 sec / 59° C, 30 sec / 72° C, 130 sec
Final elongation	1× 72°C, 7 min		
Cooling	1×, 4°C, ∞		

3.2.8.3 AAV Helper Virus PCR

To detect AAV helper viruses in some AAV-positive blood donors and immunosuppressed patient samples, 250 ng leukocytes' DNA was PCR-analyzed by the Institut Kardiale Diagnostik und Therapie (IKDT) to detect EBV, CMV and HHV-6 genomes as described before (258).

3.2.9 Purification of PCR Products

PCR products were purified using GeneMATRIX 3 in 1- Basic DNA Purification Kits according to the manufacturer's instructions. Thereafter, ≥ 75ng of purified PCR product in 15 µl 5 mM Tris-HCl, pH 8.0 were sent for sequencing. The DNA concentration was estimated by reference to the indicated size of the corresponding band on the molecular weight marker.

3.2.10 Sequencing

To determine the AAV serotype by DNA sequence analysis of PCR products, purified PCR products (see 3.2.9) were sent for DNA sequencing at Eurofins Genomics, Ebersberg, Germany. Sequences were aligned using NCBI blast at URL: http://blast.ncbi.nlm.nih.gov.

3.2.11 Cloning

Purified PCR products were cloned into pJET1.2 using the CloneJET PCR Cloning Kit (Thermo Scientific) according to the manufacturer's instructions. The quantity of PCR product used (in moles) was five times more than the vector and was calculated according to the following equation:

$$\text{DNA Quantity [pmol]} = {}^{\text{mass [pg]}}\!/_{660\ \text{[g/mol]}} \times \text{number of base pairs} \quad (259).$$

The quantity (concentration) of the vector equals 0.025 pmol, as indicated by the manufacturer. The mass was estimated according to the PCR fragment size in reference to the size of the corresponding band on the molecular weight marker. Both sticky-end and blunt-end protocols were compared. The sticky-end protocol yielded much better results and was used for all the cloned samples, suggesting that the Herculase produced PCR products with 3' dA overhang. Of the ligation mixture, 5 µl was used to transform competent E.coli and the rest was kept at -20°C in case of future need. DNA was extracted and purified from the desired clone and sent for DNA sequencing (Eurofins).

3.3 Statistical Analysis

3.3.1 Study Protocol

The study is a cross-sectional study carried out according to the Charité regulations for good scientific practice. Ethics application form was submitted by Prof. Dr Heilbronn and was granted approval by the Ethics Commission of the Charité - Universitätsmedizin Berlin. When required, the study participants signed an informed consent form provided to them with all the relevant, necessary information. Blood samples were randomly chosen from blood donors (n= 243) or immunosuppressed patients (n= 41).

3.3.2 Sample Size Determination

To obtain an estimate of the sample size needed for this study, Daniel's formula was used:

$$n = \frac{Z^2[P(1-P)]}{d^2} \quad (260)$$

where (n) is the sample size, (Z) is a statistic for the level of confidence. For a confidence interval (CI) of 95%, *"Z"* equals 1.96. *"P"* is the estimated prevalence, based on previous studies, while *"d"* is the precision. The smaller the "*d"* is, the smaller the error of estimate will be. It is double the width of CI, and in most cases, and when prevalence ranges from 10% to 90%, it is set to 0.05. Since the only study that was carried out on human blood investigating AAV-DNA prevalence was limited to a small number of subjects and only AAV-2 was tested (261), we took into consideration prevalence reported in other more recent studies carried out on a large number of human tissues and broader spectrum of AAV, though AAV was not tested in human blood in those studies (130, 84).

3.3.3 Statistical Analysis of Significance

Statistical analysis was performed using the chi-square test or Fisher's exact test.
(*P value* < 0.05 is considered significant).

4 Results

4.1 Establishment of a Highly Sensitive AAV-Specific Nested PCR Assay

Despite the ubiquity and high seroprevalence of AAV, little is known about the preferential cells for its persistence. We hypothesize that AAV persists in leukocytes. As wild-type AAV replicates and persists either in an integrated form or as an episome in the cell nucleus (73, 82-84, 70), a persistent non-replicating virus is not likely to be found free in plasma but rather to reside in nucleated blood cells, i.e. leukocytes. Therefore, PCR detection of AAV-DNA genomes had to be performed against a background of total genomic DNA extracted from leukocytes. It was important to establish an efficient PCR assay that amplifies all known human serotypes to enable a practical one-step screening of leukocytes for different AAV serotypes. The PCR assay had to be also sensitive enough to amplify a low-copy number of the virus efficiently with no need for further cloning of the PCR products to get enough pure DNA for sequence analysis, which is important to identify the serotype and confirm the sequence. The PCR had to be specific as well to avoid amplification of unspecific products, a common difficulty when amplifying templates on a background of genomic DNA.

4.1.1 Primer Design for First Round PCR

The first step in establishing PCR was to look for a conserved region of the *cap* gene of the human AAV serotypes described to date. To do that; first, the known sequences of human AAV serotypes were aligned to find a conserved region for designing primers that match all or most of human AAV serotypes. Also, previously described AAV primers (84, 262, 263) were compared, regarding the homology of their sequences to known human AAV serotypes and hence their expected hybridization efficiency, to choose or generate the most convenient primer pair. AAVsero for and rev primers were selected for PCR amplification, as they were tested before and found highly suitable to detect low copy numbers of integrated AAV2 DNA in various cell lines (262) and seemed superior to the other pairs compared (84, 263). For AAV5, the AAVsero forward primer has six mismatches, so an additional forward primer was designed (Figure 4.1). AAV5 for primer (AAV5 *nt* 2341-2364) was generated and combined with sero primers (AAVsero for (AAV2 *nt* 2350-2373) and rev primers (AAV2 *nt* 2899-2878)) previously described (262). The three primers match almost all AAV serotypes 1-13,

with a few mismatches for AAV3, 4, 9, 11, 12 and 13. To estimate PCR sensitivity and optimize PCR conditions, 1 μg of genomic HeLa DNA, roughly equivalent to $1.7x10^5$ diploid cells, was spiked with 10 plasmid copies of various AAV serotypes. PCR amplicons range between 532 bp and 553 bp for different serotypes. AAV4 was not analysed since it is not of human origin, and neither antibodies reactive to AAV4 (136, 137) nor AAV4 DNA have ever been reported in humans (130, 84, 111).

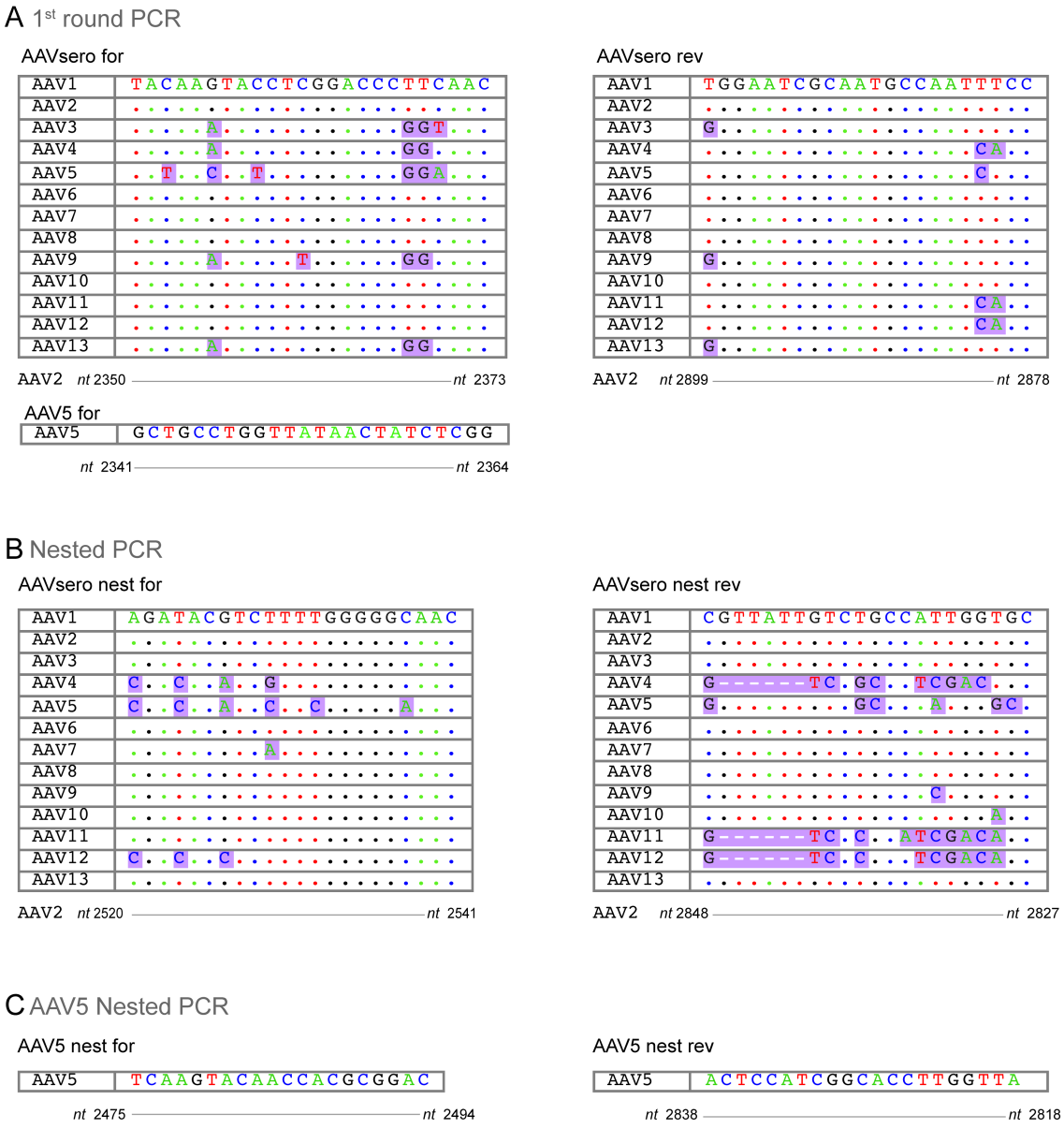


Figure 4.1 Primer mismatches.

A. First round PCR primers. Primer sequences were compared for AAV1-13 sequences. The matching nucleotides are masked, shown only as a dot of the corresponding colour (A=green, T=red, C=blue, G=black). The mismatched nucleotides are highlighted in purple. In the 1st round PCR, AAVsero For and AAVsero Rev primers bind a highly conserved region in almost all human AAV serotypes, but additional AAV5 forward primer was generated, as AAVSeroFor has 6 mismatched nucleotides for AAV5. The AAVsero Rev primer has only one mismatch for AAV5. The AAV5 forward primer sequence is displayed in a box below. **B.** Primer sequences for the nested PCR matching AAV1-3, 6-10 and 13. **C.** AAV5 nested primers. AAV5 nested PCR was carried out separately.

4.1.2 Optimization of PCR Conditions and Reaction Mixture Composition

Different primers, $MgCl_2$ and dNTPs concentration combinations, and numbers of cycles were tested to determine the optimal conditions. In addition, different polymerases were compared regarding sensitivity and specificity.

Using *Taq* DNA polymerase with ThermoPol® buffer (NEB), 10^3 copies of AAV1-3 and 5-8 were amplified under the same PCR conditions and almost the same reaction mixture as previously described except for the addition of AAV5 for primer (262). Non-template (nuclease-free water) and 1 µg genomic DNA from HeLa cells were used as negative controls to ensure purity of all reagents used for DNA extraction and PCR mixture. Unfortunately, no band was detected except for the positive control; 1 µg DNA from Detroit 6 cells containing ~$8.3x10^5$ integrated wild-type AAV2 genome, about 5 copies of virus genome / cell (264). Persistent AAV does not replicate and was not expected to be found in such a high genomic copy number in healthy blood donors, which meant more optimization of PCR was mandatory to increase PCR sensitivity (Figure 4.2).

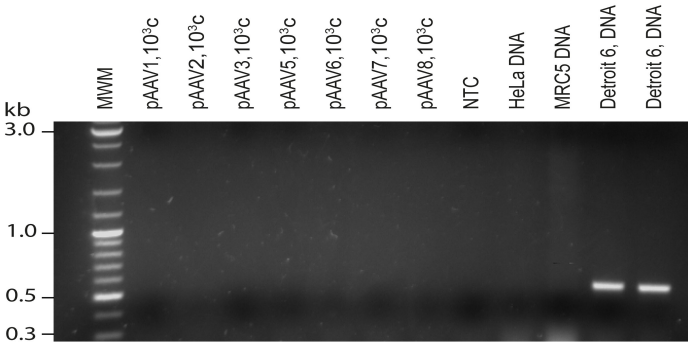
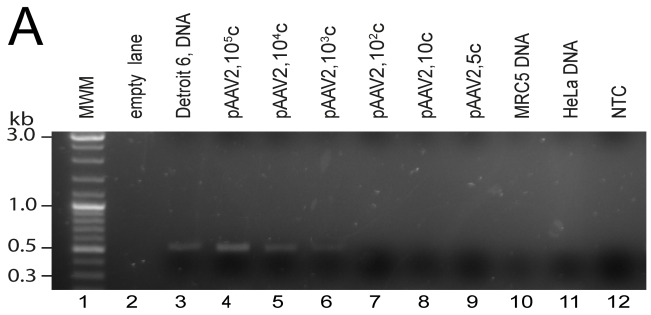


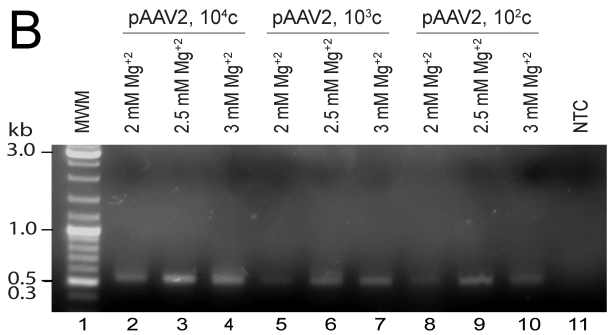
Figure 4.2 PCR testing on different AAV serotypes

Briefly, the reaction mixture was composed of primers: AAVsero for, AAVsero rev, and AAV5 for primer (0.3 µM, each), 0.2 mM dNTPs, 2 U *Taq* DNA polymerase in ThermoPol® buffer to a final volume of 50 µl. The thermocycling profiles of PCR consisted of an initial denaturation step at 94°C for 2 min, followed by 45 cycles of 94°C for 20 sec, 60°C for 30 sec, and 72°C for 1 min, then a final extension step at 72°C for 7 min. NTC=non-template control. DNA from Detroit 6 cells was used as positive control.

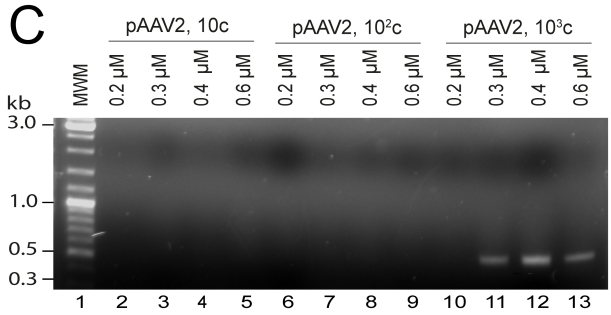
An alternative number of cycles i.e. decreased by five i.e. 40 cycles (Figure 4.3, A), different Mg^{+2} and primer concentrations (Figure 4.3, B and C, respectively), and various annealing and elongation temperatures (Figure 4.3, D and E, respectively) were tested. The threshold for AAV2 detection under the tested conditions was template containing 10^2-10^3 copies of pAAV2 (Figure 4.3, C, lanes 11-13, and E, lanes 7-14).



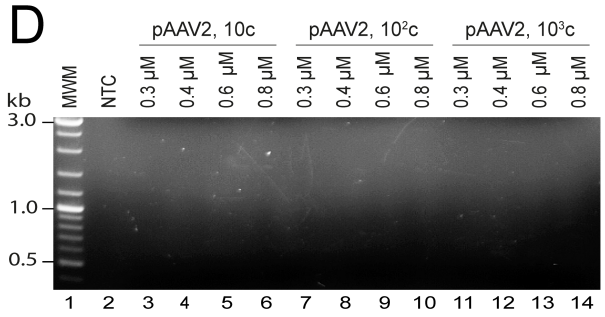
5 cycles fewer, 40 cycles



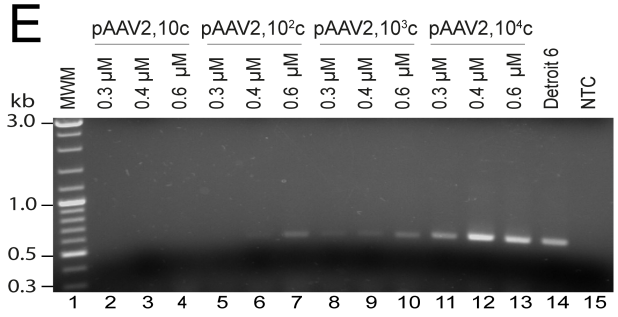
Back to 45 cycles,
optimization of Mg^{+2} concentration



Optimization of primer conc.
Mg^{+2} conc. optimized to 2.5 mM Mg^{+2}



Testing of different primer conc.
Using a lower annealing temp. of 58 °C



Testing of different primer conc.
Annealing temp. back to 60 °C.
Elongation temp. 68°C instead of 72 °C

Figure 4.3 PCR optimization through testing of different conditions and reaction mixture concentrations.

A. PCR representative gel photo. PCR was carried out on serial dilutions of AAV2 *cap* plasmid ranging from 10^5 to 5 copies to detect the sensitivity limit for the PCR under these conditions. MRC-5 DNA is extracted from a human fibroblast cell line, HeLa DNA from the HeLa cell line, and NTC is a non-template control. Faint bands could be seen in lanes 3-6, but not for the lower concentrations. **B.** Number of cycles was increased back to 45 cycles, and different Mg^{+2} concentrations were tested to determine the optimal one, which seemed to be 2.5 mM. **C.** Different primer concentrations were compared, but the 10^2 copy number was not constantly significantly amplified. **D.** Different primer concentrations were compared under lower annealing temperature to lower the stringency of PCR conditions. However, it seemed that optimal annealing temperature is a deciding factor for PCR sensitivity. **E.** The annealing temperature was brought back to 60 ºC, while the elongation temperature was lowered to 68 ºC as recommended by the manufacturer for maximal efficiency of the polymerase, while different primer concentrations were retested. Please note that the same PCR conditions as described before (see Figure 4.2) were used in all PCRs, except for A, where the number of cycles was lowered to 40 cycles.

As our goal was to achieve a sensitivity level of as low as one copy of virus genome/1 μg DNA (~1 copy /1,7 x 10^5 cells), PCR was carried out with other more sensitive high fidelity polymerases and the amplification efficiencies were compared (Figure 4.4). Platinum polymerase was the most sensitive one and permitted the detection of as few as five copies of AAV2 per 1,7 x 10^5 cells (Figure 4.4 A, lanes 6-8). It also detected different AAV serotypes (Figure 4.4 B, lanes 3-9).

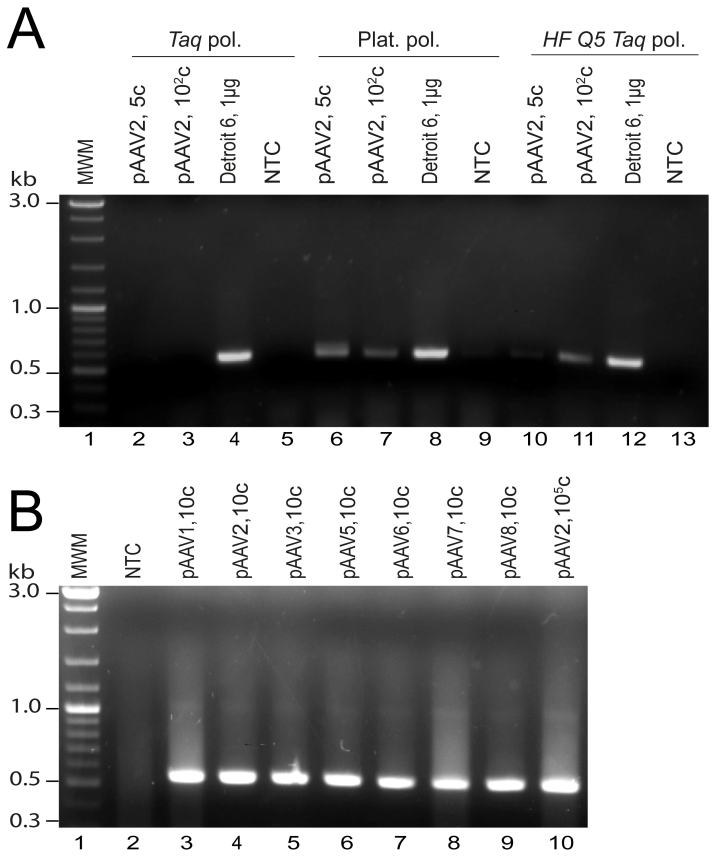


Figure 4.4 Platinum polymerase detects 10 copies of AAVs

A. Comparison of different polymerases: *Taq* polymerase with ThermoPol buffer, Platinum polymerase, and Q5 high-fidelity polymerase. Pol.= polymerase, HF= high fidelity. The reaction mixture was composed of the primers; AAVsero for, AAVsero rev, and AAV5 for primer (0.4 µM, each), 0.2 mM dNTPs, 2.5 mM Mg^{+2}, and 2 U DNA polymerase in the corresponding buffer to a final volume of 50 µl. The thermocycling profiles of PCR consisted of an initial denaturation step at 94°C for 2 min, followed by 45 cycles at 94°C for 20 sec, 60°C for 30 sec, and 68°C for 1 min, then a final extension step at 68°C for 7 min. **B.** PCR on 10 copies of different AAV *cap* carrying plasmids, using the same PCR conditions as B, except for an extension temperature of 72°C instead of 68°C. The reaction mixture was also the same except for the primer concentration (0.5 µM instead of 0.4 µM) and the Platinum polymerase (2 U instead of 1 U per reaction). All plasmids were spiked on 200 ng genomic HeLa DNA.

The next DNA samples from 105 healthy blood donors were screened using Platinum polymerase according to the optimized protocol mentioned above (same for plasmids in Figure 4.4, B). The PCR conditions were sensitive enough for amplification of 10^2 copies of AAV-5 and 10 copies of AAV2 (Figure 4.5, A, lane 17; and B, lane 11), but all tested donors' samples were negative (Figure 4.5, A, lanes 2-14 and B, lanes 2-9). A question was raised as

to whether these samples would have been entirely negative or if the PCR had to be further optimized.

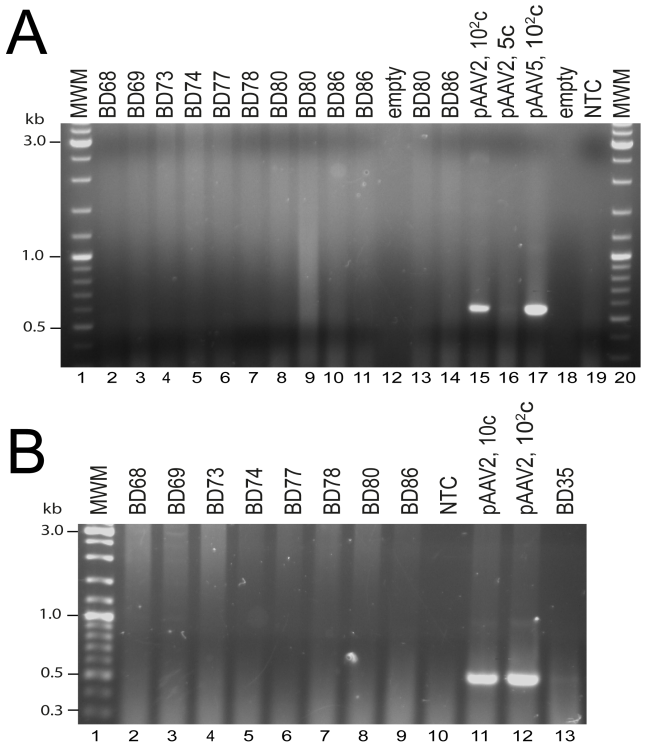


Figure 4.5 Screening of genomic DNA from 105 blood donors using Platinum polymerase.

A. and **B.** Two representative gel photos of PCR carried out using Platinum polymerase on 1 µg leukocytes DNA from 105 healthy blood donors. The expected lengths of the amplicons range between 532 and 553 bp. MWM = molecular weight marker; pAAV2, 10 c and 10^2 c depict the positive control (1 µg genomic DNA spiked with 10 plasmid copies of AAV2); NTC = non-template control.

To settle that question, a more sensitive proofreading polymerase, Herculase, was tested in PCR with different primer concentrations. PCR was carried out on two samples and 10 copies of pAAV2 spiked on 1 µg genomic DNA from buffy coat (Figure 4.6). AAV2 genome in leukocytes DNA could be detected only after increasing the primer concentration to 0.6 µM (Figure 4.6, lanes 5 and 9).

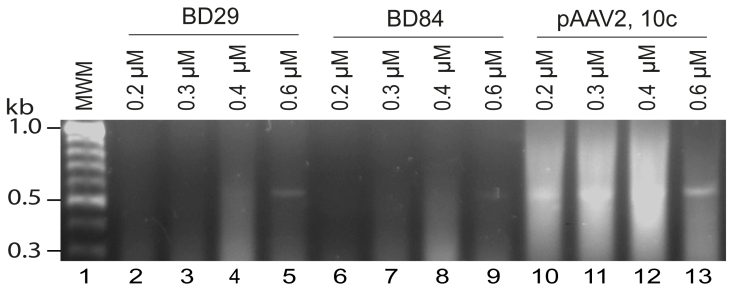


Figure 4.6 Detection of AAV2 in blood samples requires Herculase and 0.6 µM primer concentration.

Primer concentrations between 0.2 and 0.6 µM were tested in PCR carried out on two blood samples. Ten copies of pAAV2 were used for positive control.

Herculase was used to amplify 10 copies of AAV *cap*-carrying plasmids AAV1-3 and AAV5-13 spiked on a background of 1 µg HeLa DNA (Figure 4.8, B). All serotypes tested showed a well-defined specific band of expected molecular weight. As few as two copies of AAV2 and AAV5 on a background of 1 µg genomic DNA were used as templates for PCR and could also be amplified (Figure 4.8, C). For this reason, the next 1st round PCR analysis was carried out with Herculase under the optimized conditions with 0.6 µM primers allowing the detection of different types of AAV in human leukocytes (see sections 4.2.1 and 4.3.1).

4.1.3 Nested PCR

The 1st round PCR could detect as few as two copies of AAV plasmid on the background of 1 µg genomic DNA, corresponding to 1.7 x 10^5 cells (Figure 4.8, C). To ensure specificity of the amplicon and determine the concerned AAV serotype, PCR products had to be sequence-analysed, which meant a much larger amount of pure amplicon was needed. For this reason and to increase the PCR sensitivity, nested PCR was developed. To be able to further amplify as many serotypes of human AAV as possible, the known human AAV serotypes were aligned using ClustalW2, and a highly conserved region was found within the 1st round PCR amplicon sequence. A pair of nested primers, AAVsero nest for (AAV2 *nt* 2520-2541) and AAVsero nest rev (AAV2 *nt* 2848-2827) was generated that amplifies AAV1-3, 6-10 and 13 (Figure 4.1, B). AAVsero nest forward and reverse primers' sequences are 100% identical to AAV1-3, 6, 8, and 13, and show a single mismatch in the forward primer for AAV7 and in the reverse primer for AAV9 and 10. The nested reverse primer showed mismatches of more

than 10 bases for AAV4, 11 and 12 (Figure 4.1). These AAVs are mostly non-human serotypes, as they were isolated initially from NHP tissues or simian adenovirus stock. They have not been detected to date in humans (123, 131, 132) and no reactive antibodies against those serotypes have yet been reported or characterised (136, 137, 126, 132, 133), so no additional matching primers were designed. The expected PCR products range from 329 to 332 bp for different AAV serotypes. As mentioned before, AAV5 is the most divergent serotype, so a separate nested PCR for AAV5 had to be developed. Nested AAV5 primer pair was generated (AAV5 nest for (*nt* 2475-2494) and AAV5 nest rev (*nt* 2838-2818)), leading to an amplicon of 364 bp (Figure 4.8, D, E). Nested AAV5 PCR was carried out separately.

Touchdown PCR was first described to overcome unspecific amplification (265). The principle is to start with an annealing temperature higher than the expected optimal annealing temperature, incrementally decreasing in subsequent cycles to a touchdown at ~15°C lower temperature. The optimal annealing temperature (T_a^{opt}) was calculated using the following formula:

$$T_a^{opt} = 0.3 \times T_m^{Primer} + 0.7 \times T_m^{Product} - 14.9,$$

where T_m^{Primer} and $T_m^{Product}$ are the melting temperatures of the primer and the PCR product, respectively (266).

Another advantage of touchdown PCR is that even using non-degenerate primers with certain mismatches, it can still amplify single specific amplicon under standard conditions (267). The annealing temperature was raised three degrees above the calculated optimal annealing temperature in the first eight cycles, then it was lowered to the optimal annealing temperature in the next ten cycles. In the next 17 cycles, the annealing temperature was further reduced to decrease the stringency of PCR conditions and help further amplification of the specific product. The optimized nested AAV PCR assay reached a sensitivity limit of as low as one to five copies of AAV genome per ~10^5 cells and was specific enough that no unspecific products or smear were detected (Figure 4.8, D and E). The strength of the signal in the 1st round PCR was equally strong for all serotypes except for AAV3 and 9, where the signal was somewhat weaker (Figure 4.8, B). In the nested PCR, the signal was equally strong for all serotypes, except for AAV11 and 12, which showed no bands because of primer mismatches, as mentioned before (Figure 4.8, D). Using this optimized nested AAV assay, DNA extracted from blood samples was screened for the presence of AAV-DNA. Protocol summarized in Figure 4.7.

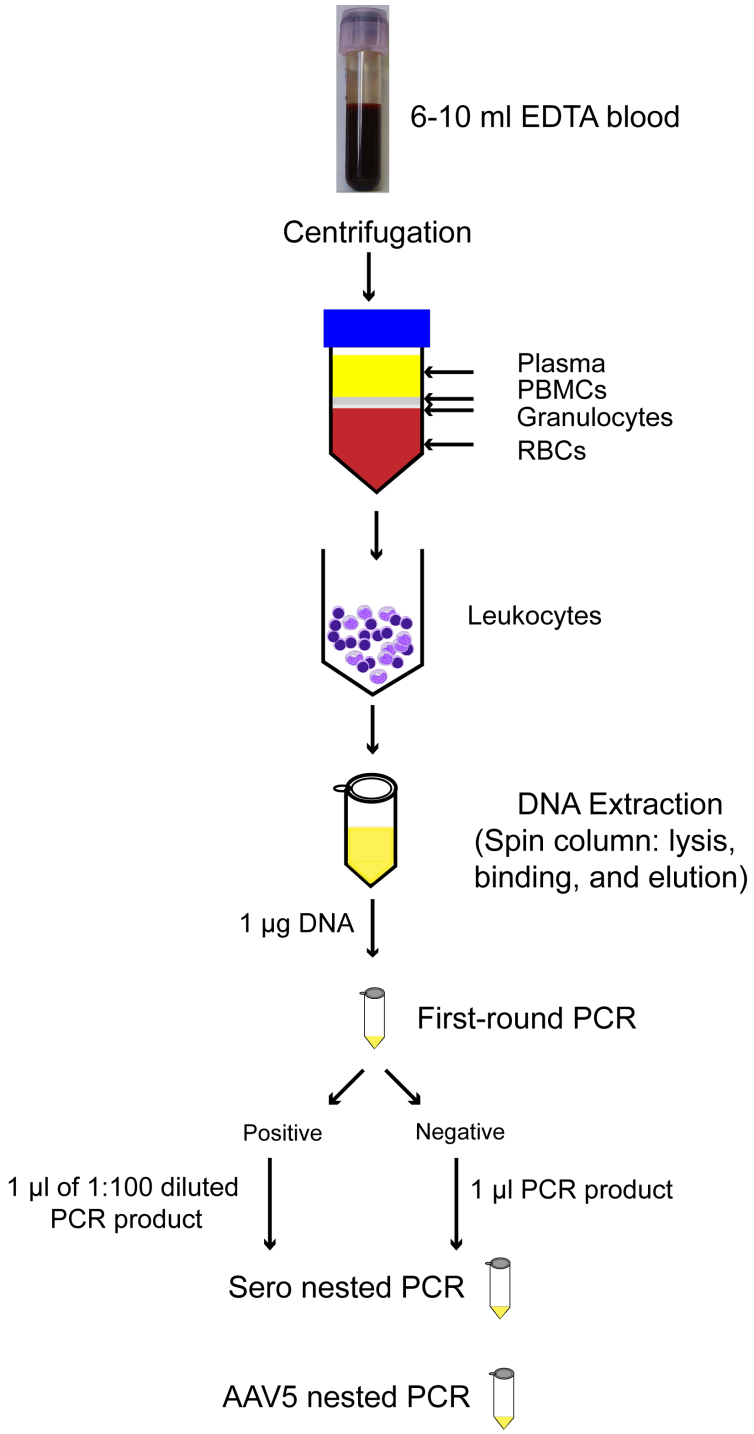


Figure 4.7 AAV PCR Assay Workup Scheme

Schematic representation of how the blood samples were processed for DNA extraction, followed by PCR analysis for the detection of AAV-DNA. PBMCs stands for peripheral blood mononuclear cells.

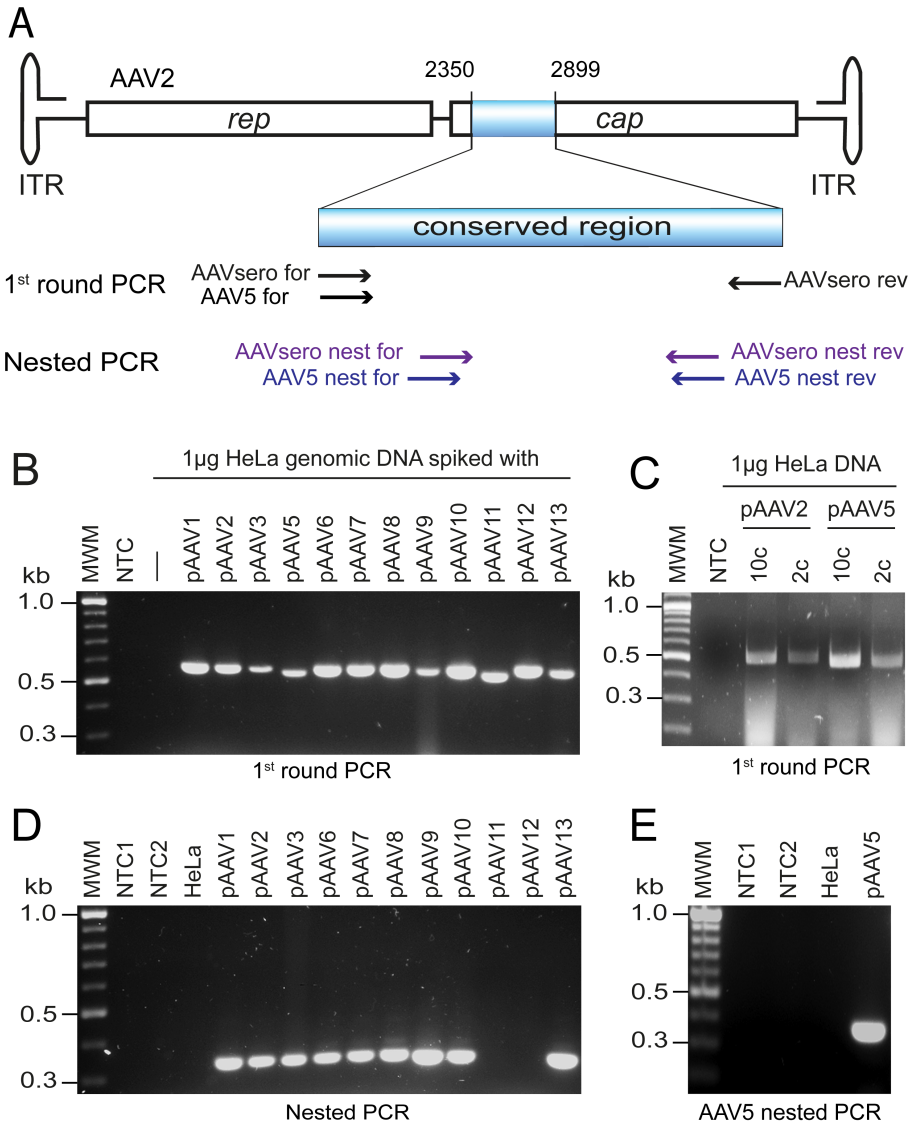
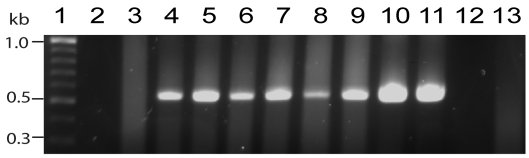


Figure 4.8 Highly sensitive nested PCR for AAV serotypes 1-13.

A. Schematic representation of the AAV genome with *rep* and *cap* genes flanked by inverted terminal repeats (ITR). A highly conserved region of the *cap* gene (*nt* 2350 - 2899) of AAV2 is blown up. All primers are depicted by arrows. AAVsero for, AAVsero rev and AAV5 for primer are used to amplify AAV 1-3, and AAV5-13). AAVsero nest for and AAVsero nest rev bind to AAV1-3, 6-10 and 13. AAV5 nested primers are used in a separate PCR to amplify AAV5. **B.** Gel photo of 1st round PCR on 1µg genomic DNA from HeLa alone or spiked with 10 copies of plasmids of various AAV serotypes. MWM = molecular weight marker, NTC = non-template control. The 1st round PCR product ranges between 532 bp and 553 bp, depending on the serotype. **C.** 1st round PCR performed as in **B** but on two copies of AAV2 or AAV5 *cap*-carrying plasmids. **D.** Nested PCR with sero nested primers. NTC1 denotes DNase-free water; NTC2 denotes amplification of non-template control of 1st round PCR. Product lengths range from 329 to 332 bp. **E.** AAV5 nested PCR with AAV5nest for and AAV5nest rev primers amplifying a product of 364 bp. This figure was previously published in Hüser, Khalid et al. J Virol 2016, Dec 7. pii: JVI.02137-16

It is well known that blood may contain PCR inhibitors, which may hinder the sensitivity of PCR (268). To investigate this concern, 10, 10^2 and 10^5 copies of pTAV-2.0 (wild-type AAV2 plasmid) alone and spiked on 250 ng –1 µg genomic DNA extracted from blindly chosen buffy coat samples and from HeLa cells were used as templates for PCR and the signal intensity was compared. No striking difference in signal intensity was noticed between samples analysed on the background of buffy coat DNA, HeLa DNA or H_2O, confirming the purity of the extracted leukocyte DNA and lack of inhibitory materials (Figure 4.9).



1-MWM
2-empty lane
3-1µg DNA, HeLa
4-pAAV2, 10c
5-pAAV2, 10^2c
6-pAAV2, 10c, spiked on 250 ng leucocytes‘ DNA
7-pAAV2, 10^2c, spiked on 250 ng leucocytes‘ DNA
8-pAAV2, 10c, spiked on 1µg leucocytes‘ DNA
9-pAAV2, 10^2c, spiked on 1µg leucocytes‘ DNA
10-pAAV2, 10^5c, spiked on 250 ng leucocytes‘ DNA
11-pAAV2, 10^5c, spiked on 300 ng DNA from HeLa
12-empty lane
13-NTC

Figure 4.9 Testing for inhibitory materials.

PCR was carried out on control plasmids, alone and spiked on 250 ng–1 µg genomic DNA extracted from blindly chosen buffy coat samples and from HeLa cells.

4.2 AAV in Leukocytes of Blood Donors

4.2.1 AAV Prevalence in Leukocytes of Blood Donors

Using the AAV PCR assay, DNA extracted from leukocytes of 243 healthy blood donors was screened for the presence of AAV-DNA. PCR results were only considered when at least two negative controls (H_2O; HeLa DNA) and two positive controls (10 copies of AAV *cap* plasmids spiked on genomic DNA) reacted as expected. For 225 blood samples from healthy blood donors, 1 µg of genomic DNA was analysed, while for the other 18 samples with lower DNA yields, only 0.3 to 0.9 µg was used as templates for PCR. A series of seven AAV-positive samples was retested once more, confirming PCR reproducibility (Figure 4.10).

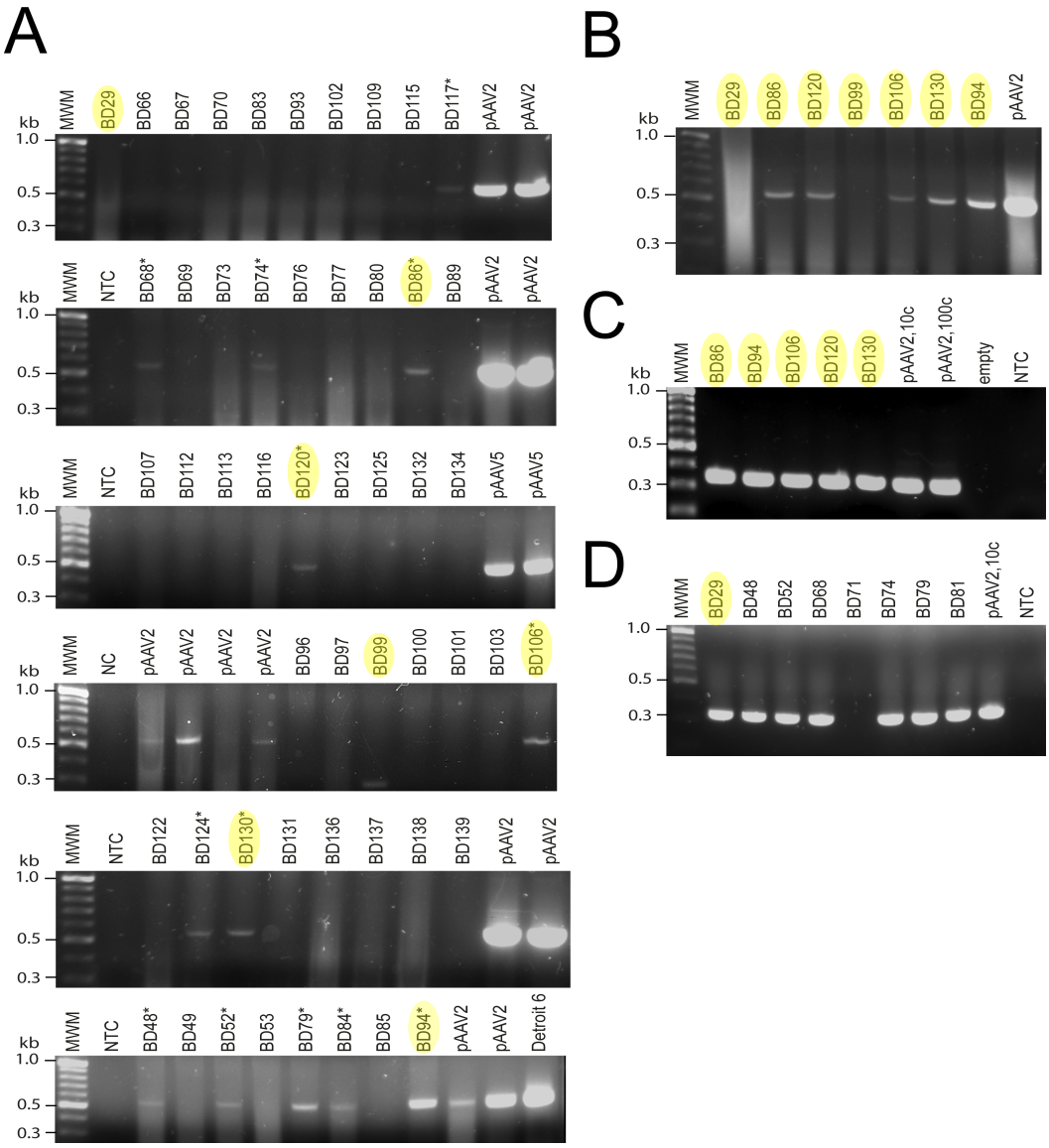


Figure 4.10 Reproducibility of PCR.

A. Gel photos of 1st round PCR carried out on some blood donors' samples to confirm PCR reproducibility, where the retested samples are highlighted in yellow. MWM = molecular weight marker, NTC = non-template control, NT = negative control (1 µg genomic DNA). Positive controls pAAV2 and Detroit 6 denote 1 µg genomic DNA spiked with 10 plasmid copies of AAV2, and 1 µg genomic DNA from Detroit 6 cell line latently infected with wt-AAV2, respectively. **B**. First round PCR repeated for seven blood samples to test PCR reproducibility. Asterisks denote positive samples. **C., D.** Nested PCR for the seven samples. Sample BD29 showed a smear in repeated 1st round PCR, however, in nested PCR a clear, well-defined band of the expected weight length was observed. Sequence analysis confirmed the specificity of the PCR product for all samples. BD71 was used as a negative control, as it did not show any band in first round PCR.

In 1st round PCR, 42 samples (17%) out of 243 contained a detectable level of AAV-DNA. AAV sero nested, and AAV5 nested PCR were carried out with the primer pairs AAV sero nested or AAV5 nested, respectively, for all samples. In AAV5 nested PCR, 16 samples tested positive. In AAV sero nested PCR, 75 tested positive (representative gel photos are shown in Figure 4.11). All samples that showed signals in 1st round PCR were also positive in AAV sero nested PCR, except for one (BD19), which tested positive in AAV5 nested PCR instead, and on sequence analysis contained exclusively AAV5 DNA (Figure 4.11, A-C). In addition, another 34 samples, apparently negative in 1st round PCR, showed an AAV-specific band in AAV sero nested PCR confirmed with sequence analysis (representative gel photos were presented in Figure 4.11, A-C). Considering mixed infection, a total of 83 out of the 243 blood donor samples screened contained AAV-DNA (34%) (Figure 4.11, D).

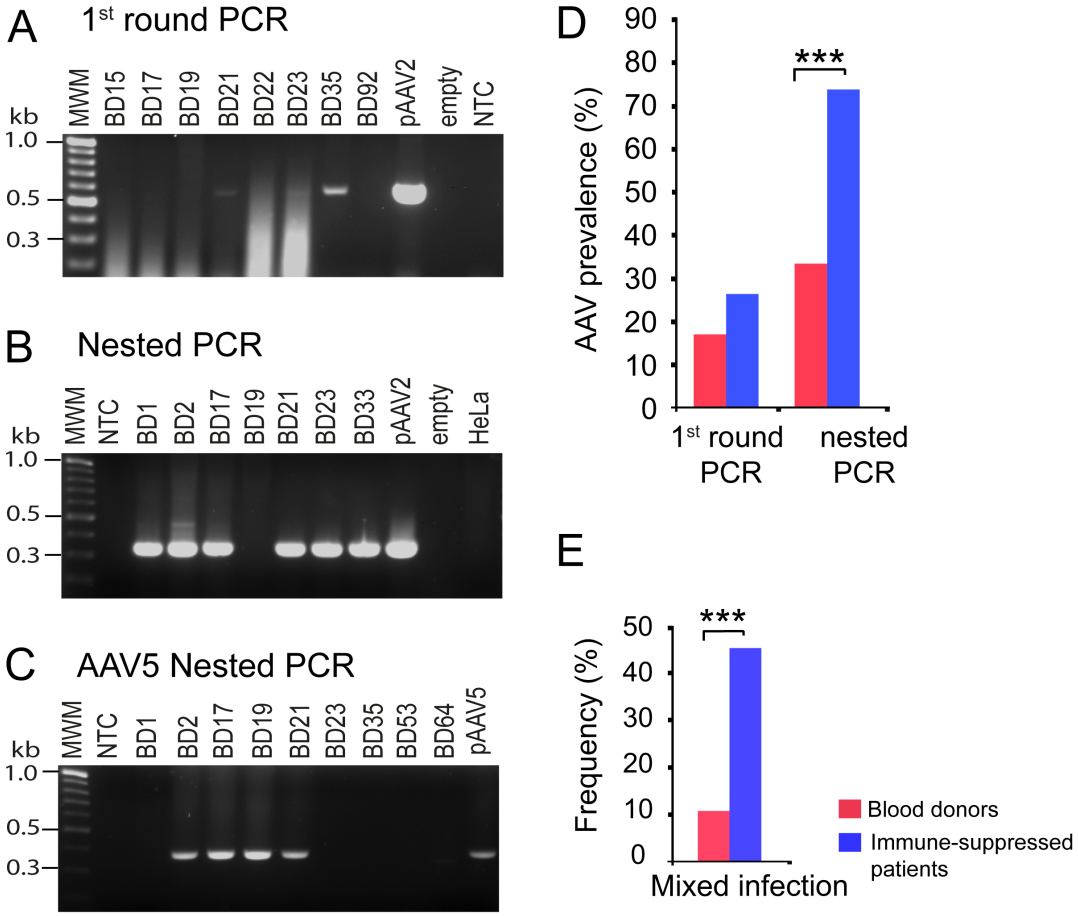


Figure 4.11 Detection of AAV in PBMCs of blood donors.

A. Results of 1st round PCR carried out on 1 µg genomic DNA extracted from PBMCs of blood donors as depicted by numbers. A representative gel is displayed showing amplicons of the expected lengths of 532 to 553 bp. MWM = molecular weight marker; pAAV2/pAAV5 depict the positive controls, 1 µg genomic DNA spiked with 10 plasmid copies of AAV2 or AAV5, respectively; NTC = non-template control. **B.** Representative gel of nested PCR with AAVsero nest for and AAVsero nest rev primers on blood donors' samples. The nested PCR products range between 329 and 332 bp. For positive samples and positive controls, 1 µl of 10^{-2} diluted 1st round PCR product was used as template, while for samples that did not show any signal in the 1st round PCR as well as negative controls, 1 µl was used. In addition, 1 µl "new" non-template negative control was tested to exclude contamination in either of the PCR rounds. **C.** Representative gel of AAV5 nested PCR products, 364 bp. **D.** Prevalence of AAV detected by 1st round and nested PCR in healthy blood donors compared to immunosuppressed patients. ***: Highly significant ($p < 0.00001$). **E.** Frequency (%) of mixed infections with more than one AAV serotype in leukocytes of tested blood donors and immune-suppressed patients. ***: highly significant ($p<0.0001$). This figure was previously published in Hüser, Khalid et al. J Virol 2016, Dec 7. pii: JVI.02137-16

4.2.2 AAV Serotypes in Leukocytes of Blood Donors

To identify the AAV serotype concerned, PCR fragments of all AAV-positive samples were column-purified and submitted for DNA sequence analysis. As displayed in Figure 4.12, 64 samples (77%) contained AAV2, 58 of them as a single isolate, while the other six samples contained both AAV2 DNA and other AAV serotypes. AAV5 DNA was found in 16 samples (19%), eight of them containing AAV5 DNA only, while the other eight had mixed infections with other AAV serotypes. AAV1 DNA was found in three samples, one of them having AAV5 DNA as well (Table 4.1).

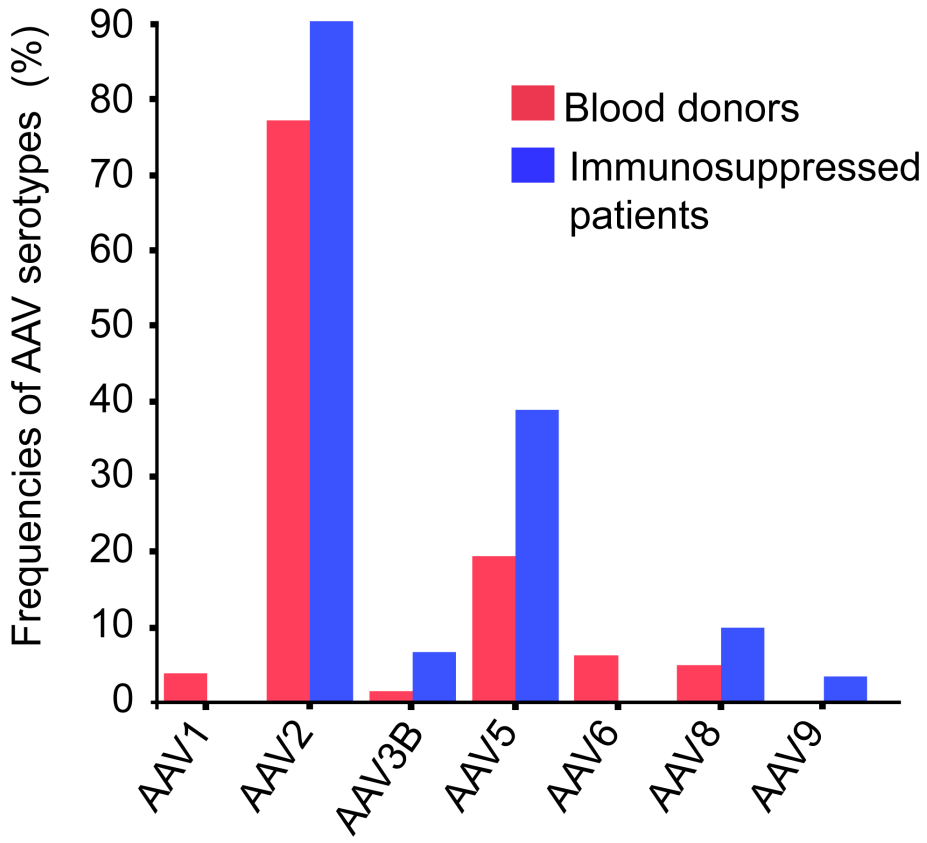


Figure 4.12 Relative distribution of AAV serotypes in blood donors and immunosuppressed patients.

AAV serotypes were identified by DNA sequence analysis of purified PCR products. The overall distribution of different serotypes among immunosuppressed patients and blood donors is similar to a great extent, being generally higher in immunosuppressed patients, with the exception of AAV1/AAV6, which were totally missing in immunosuppressed patients. This figure was previously published in Hüser, Khalid et al. J Virol 2016, Dec 7. pii: JVI.02137-16

Other AAV isolates with 95-100% DNA sequence identity to AAV1, 3B, 6 and 8 were detected (Table 4.1). Most isolated AAV sequences share 99-100% sequence similarity with wild-type human AAV most homologous serotype, as the amplicon locates in a relatively conserved region of the *cap* gene. Interestingly, all AAV1 sequences isolated from these samples share a mutation at position 2,774 *nt,* where T is substituted for C when aligned against the wild-type AAV1 published sequence (NC_002077.1).

In a trial to interpret the possible significance of this mutation, the sequence of PCR product was assembled on the most identical wild-type AAV sequence (AAV1) using Gene Construction Kit program GCK only replacing the corresponding sequence with the PCR product sequence having the mutation. Interpretation of this mutation revealed that it is a silent mutation for open reading frame 1 (ORF1) coding for VP1, but it codes for a different amino acid (threonine instead of isoleucine) for ORF2 coding for Assembly Activating Protein (AAP). For another isolate (BD149), which has two additional mutations, assembly on the published wild-type AAV1 sequence using GCK only replacing the region of PCR amplicon denoted silent mutation for ORF2, but coding for different amino acids for ORF1 (Table 4.2). Of note is that some of the mutations already lie within the hypervariable regions (HVR1 and 2) described before (269), while others are scattered.

A sequence that shares 97% identity with AAV3B-DNA was identified once (BD75). At the level of protein, almost all the different nucleotides code for the same amino acid as for wild-type AAV3B, except for one in ORF1 coding for VP1 and another one in ORF2 coding for AAP. AAV6-DNA was isolated from five samples, where three samples are homologous with wild-type AAV6, while the other two share 96%-98% sequence similarity with AAV6. One isolated sequence from sample BD142 shares 96% sequence identity with AAV6, where the mutated nucleotides code for four different amino acids in VP1 and eight in AAP, while the other mutations are silent (Table 4.2). AAV8 was detected in four samples, three of them containing AAV5 DNA as well, while the fourth one (BD35) was the sole isolate and shared 95% sequence identity with AAV8 and other isolates of the previously described clade E (130). PCR was repeated for sample BD35 and BD224, and sequence analysis revealed repeated detection of the same mutations, excluding PCR- or sequencing- induced error. Mixed infections with more than one AAV serotype accounted for 11% (Figure 4.11, E). In total, 93 AAV sequences were isolated from 83 AAV-positive blood donors' samples. Interestingly, all the mutations in these sequences lie downstream from phospholipase coding region of the *cap* gene. The region of isolated sequences coding for phospholipase is highly conserved. Phospholipase is reported to be essential for AAV infectivity (61).

Table 4.1 Distribution of AAV serotypes detected as single isolates and mixed with other serotypes in blood donors and immunosuppressed patients.

BD / IS	AAV1		AAV2		AAV3		AAV5		AAV6		AAV8		AAV9	
Single isolate	2	-	58	14	1	-	8	2	4	-	1	1	-	-
Mixed infection with an/other serotype/s	1(AAV5)	-	4(AAV5) 1(AAV6) 1(AAV5, 8)	1(AAV3B) 1(AAV3B, 5) 9(AAV5) 2(AAV8) 1(AAV9)	-	1(AAV2) 1(AAV2, 5)	1(AAV1) 4(AAV2) 2(AAV8) 1(AAV2,8)	9(AAV2) 1(AAV2, 3B)	1(AAV2)	-	2(AAV5) 1(AAV2, 5)	2(AAV2)	-	1(AAV2)
Total isolates	3	-	64	28	1	2	16	12	5	-	4	3	-	1

Blood donors' isolates (BD) are shown in red, and immunosuppressed patients' isolates (IS) are written in blue. The number before the brackets indicates the number of isolates of certain serotype mixed with another AAV serotype (shown in the brackets). This table was previously published in Hüser, Khalid et al. J Virol 2016, Dec 7. pii: JVI.02137-16

Table 4.2 Sample mutations interpretation.

Sample no.	Maximum homologous serotype	Novel amino acids		Silent amino acids	
		VP1	AAP	VP1	AAP
BD35	AAV8, 98%	Q84K*, R169K*, L189I*, A195G, V199L*, P201S, N202G	F7C, Q8P, L12Q, L16P, Q17P, R19A, I26V	82, 165, 168, 170, 173, 175, 183, 184, 188, 193	-
BD35	AAV10, 97%	D24A, A135G, E179D*	S3T*, S7C, H16P, Q17P, L34H, Q50R*	17, 18, 165, 168, 169, 170, 173, 175, 183, 184, 188, 193, 195, 198, 200, 201, 202	8, 12, 19, 22, 24, 25, 26
BD75	AAV3B, 98%	I125V*, P195S	H16R	113, 121, 190	-
BD142	AAV6, 96%	S149P, S179A*, E180D*, E190Q*	A2E, S5T*, S7Y, P8L, H10P, N11S*, E14D*, N15S*	177, 182, 183, 185, 186	3
BD149	AAV1, 98%	S156P, F173S	I9T	184	-
BD224	AAV2, 97%	V151A, Q164N, T205S*	Q6P	77, 121, 134, 145, 161, 169, 173, 181	29

BD denotes blood donors. VP1 denotes capsid protein, AAP Assembly Activating Protein. The numbers denote the position of the amino acid in the corresponding protein of the most homologous AAV serotype and the letter before and after are the one-letter abbreviations stand for the original and mutated amino acids, respectively. * Indicates a missense mutation that leads to a functionally similar amino acid.

4.2.3 Persistence of AAV Infection

The high prevalence of AAV infection in healthy blood donors accompanied by frequent mixed infections supports our assumption of persistent AAV infection in leukocytes. To give further evidence to this postulation, some AAV-positive blood donors were repeatedly screened over a follow-up period of ~ 24 months. Nineteen blood donors were tested twice, and 42% were confirmed as AAV-positive (n = 19) (Table 4.3), whereas 33%, of those tested for a third time were positive again (n = 6) (Figure 4.13). Two blood donors could be tested four times, and both were repeatedly positive.

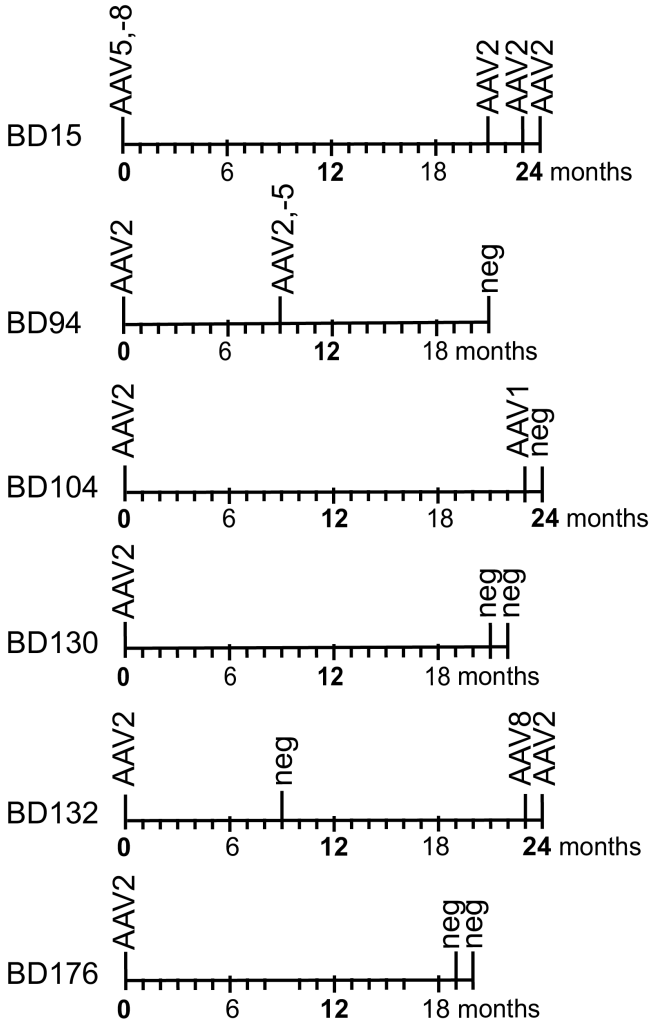


Figure 4.13 Follow-up of AAV persistence over 24 months.

Timeline and PCR results of six blood donors tested three or four times are displayed. The donors' anonymous code is given on the left side. The serotypes identified are depicted at the respective time point. "neg" indicates no detectable level of AAV-DNA at the respective time point. This figure was previously published in Hüser, Khalid et al. J Virol 2016, Dec 7. pii: JVI.02137-16

Table 4.3 Follow-up PCR of some positive blood donors.

Sample number	1st time testing result	Serotype	Time interval (months)	2nd time testing result	Serotype
BD2	Positive	AAV2, 5	10	Positive	AAV2, 5
BD4	Positive	AAV5	24	Negative	-
BD15	Positive	AAV5, 8	21	Positive	AAV2
BD19	Positive	AAV5	10	Negative	-
BD21	Positive	AAV2, 5	22	Negative	-
BD22	Positive	AAV6	24	Negative	-
BD33	Positive	AAV2	9	Positive	AAV2
BD35	Positive	AAV8*	9	Negative	-
BD48	Positive	AAV2	9	Positive	AAV1
BD89	Positive	AAV2	23	Negative	-
BD94	Positive	AAV2	9	Positive	AAV2, 5
BD104	Positive	AAV2	23	Positive	AAV1
BD106	Positive	AAV2	9	Negative	-
BD130	Positive	AAV2	21	Negative	-
BD132	Positive	AAV2	9	Negative	-
BD138	Positive	AAV5	21	Negative	-
BD176	Positive	AAV2	19	Negative	-
BD244	Positive	AAV2	1	Positive	AAV2
BD258	Positive	AAV2	2	Positive	AAV2, 5

Nineteen positive blood donors were tested twice. The serotype detected each time is shown. * Indicates ≤95% homology with wild-type AAV. This table was previously published in Hüser, Khalid et al. J Virol 2016, Dec 7. pii: JVI.02137-16

4.3 AAV in Leukocytes of Immunosuppressed Patients

4.3.1 AAV Prevalence in Leukocytes of Immunosuppressed Patients

Many of AAV helper viruses, including CMV (9), EBV (8) and HHV-6 (10), are known to persist latently, and replicate in cells of hematopoietic origin if the conditions are permissive (107-111). They are frequently reactivated under immunosuppression with poorer prognosis (112-115). Reactivation of its helper viruses should be accompanied by active replication of persistent quiescent AAV and subsequently a higher detection rate. This prompted us to investigate AAV prevalence in PBMCs of immunosuppressed patients (See 3.2.7.4), encouraged by the high prevalence of AAV-DNA in PBMCs of healthy blood donors observed in this study. Genomic DNA was extracted from buffy coat samples of 41 post-transplant patients under immunosuppressive therapy. The samples were PCR-analysed for AAV-DNA using a nested AAV PCR assay (representative gel photos: Figure 4.14). In the 1st round PCR, 11 out of 41 samples (27%) contained a detectable level of AAV-DNA. Nested AAVsero PCR and nested AAV5 PCR were carried out on all the 1st round PCR products; 29 and 12 scored positive, respectively (representative gel photos: Figure 4.14, B and C). Forty-six AAV sequences, 17 as single isolates and 29 mixed with other AAV serotypes, were isolated from 31 positive patients (Table 4.1). Taken together, AAV prevalence almost doubled in immunosuppressed patients compared to healthy blood donors (76% vs. 34%, p <0.000001) (Figure 4.11, D). The percentage of mixed infections was also significantly higher in immunosuppressed patients than in healthy blood donors, 45% vs. 11% (Figure 4.11, E).

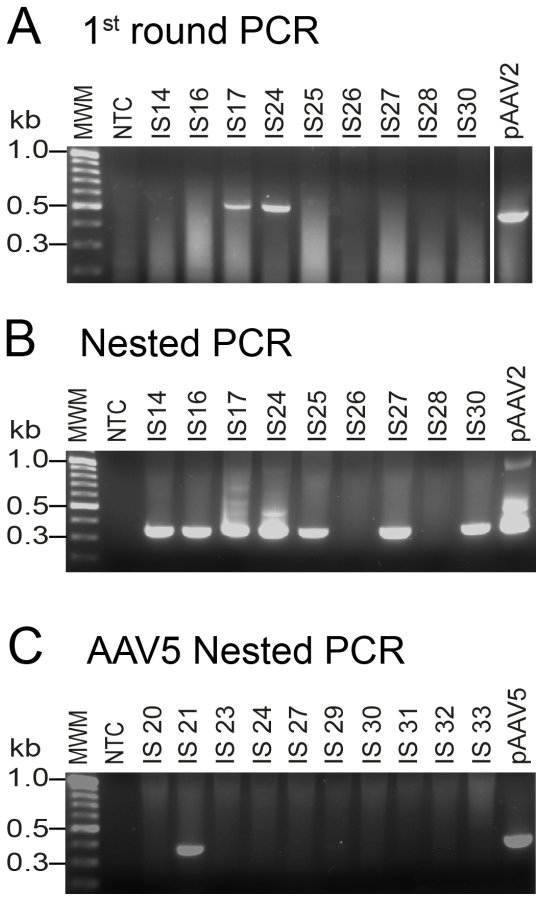


Figure 4.14 Detection of AAV in PBMCs of immunosuppressed patients.

PBMCs DNA from 41 immunosuppressed patients was tested for the presence of AAV-DNA using AAV PCR assay as described in 3.2.8.1. **A.** Representative gel of 1st round PCR. 1 µg genomic DNA extracted from PBMCs from 41 immunosuppressed patients as depicted by numbers. Lengths of different amplicons range between 532 and 553 bp. MWM = molecular weight marker; pAAV2/pAAV5 depict the positive controls; 1 µg genomic DNA spiked with 10 plasmid copies of AAV2 or AAV5, respectively. NTC= non-template control. **B.** Representative gel of nested PCR with AAVsero nest for and AAVsero nest rev primers on immunosuppressed patients' samples. The nested PCR products range between 329 and 332 bp. The template for nested PCR is the same as for blood donors, either diluted or non-diluted 1st round PCR products. **C.** Representative gel of AAV5 nested PCR. PCR product is 364 bp. This figure was previously published in Hüser, Khalid et al. J Virol 2016, Dec 7. pii: JVI.02137-16

4.3.2 AAV Serotypes in Leukocytes of Immunosuppressed Patients

AAV2 was the most abundant serotype, detected in 28 samples (90%), followed by AAV5 (39%). Whereas AAV3B and AAV8 were occasionally detected, AAV1 and 6 were not detected in any of the screened samples (Figure 4.12). Half of AAV2 isolates (n = 14)

were detected as single isolates, while the other half (n = 14) was mixed with other AAV serotypes. One of them (IS10) also had AAV3B DNA as a co-infecting serotype. In another nine of these AAV2-positive samples, AAV5 sequence was detected as well, whereas in another sample (IS21) additional AAV3B and AAV5 DNA sequences were identified (Table 4.1). In two patients' samples AAV5 DNA was the sole AAV serotype recovered, so a total of 12 AAV5 isolates were identified. AAV8 DNA was isolated from three samples, in one of which the AAV8 sequence was the only isolate found, while in the other two samples, AAV2 DNA was also detected. One of these isolated sequences has < 96% homology with wild-type AAV8 and closely related previous human isolates, hu.6 and hu.17, (i.e. the previously described clade E(130)). AAV9 was detected only once.

4.4 Cloning of Some Purified PCR Products from Blood Donors and Patients.

As the primers we generated bind almost all human AAV serotypes, it is possible, in the case of mixed AAV infection, that one serotype might be amplified in a privileged way. In other words, mixed infection with more than one AAV serotype might be underestimated, as they could not be distinguished with the consensus human AAV primers we used. To investigate this possibility, all available raw sequencing data of purified PCR products of blood donors and immune-suppressed patients were thoroughly revised. Most samples' raw data showed uniform, distinctive, single, evenly spaced peaks. However, some isolated sequences, especially from immunosuppressed patients, showed overlapping peaks (Figure 4.15). Cloning of purified PCR products from three blood donors and seven immunosuppressed patients, whose raw data trace showed overlapping superimposed peaks, was carried out. Different clones were analysed by restriction digestion with XbaI / XhoI to confirm ligation of the PCR product. As most of the cloned samples had AAV2 as a dominant serotype (six out of ten), the clones generated from these samples underwent restriction analysis using RsaI, which cuts once within AAV2 PCR amplicon region (*nt* 2350-2899), but not in most other serotypes. AAV9 is an exception, but none of our cloned samples contained AAV9 as a dominant serotype. Some clones that were digested with RsaI were submitted to sequence analysis and confirmed to be AAV2. Sequence analysis of multiple clones from each sample confirmed our postulation. Various additional serotypes, apart from the initially isolated serotype, were isolated (Table 4.4). This means that the prevalence of certain AAVs may be underestimated in the case of mixed infections. However, the distribution of AAV serotypes

among immunosuppressed patients and healthy blood donors is invariably similar and consistent with the reported AAV seroprevalence.

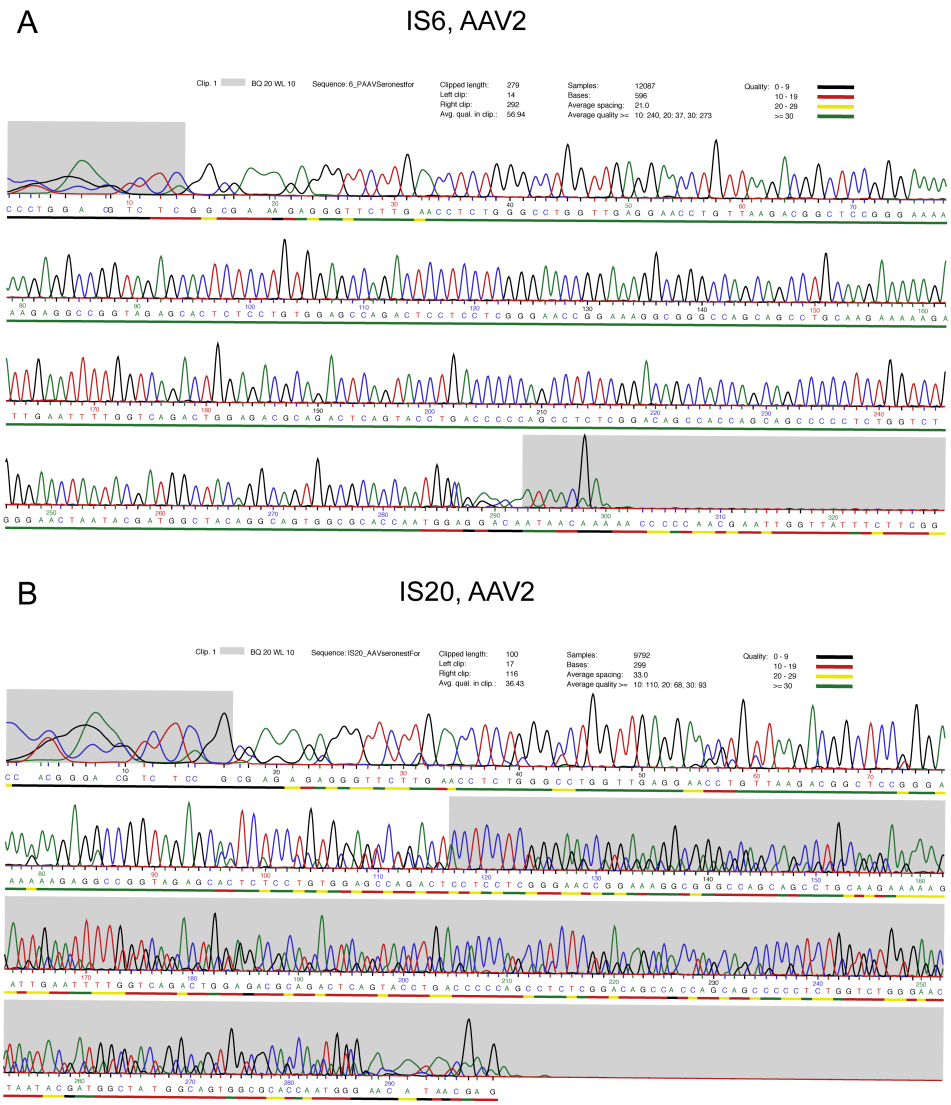


Figure 4.15 Raw sequences of two immunosuppressed patients' samples.

Analysis of both sequences revealed the presence of AAV2. However, by revision of available raw sequences data, some samples, such as IS20 in B. showed two overlapping sequences. Cloning of purified PCR products of these samples revealed in most cases the presence of additional AAV serotype/s. Purified PCR products were sequenced by Eurofins Genomics.

Table 4.4 AAV serotypes detected by cloning of some blood donors' and immunosuppressed patients' samples.

Sample No.	Dominant serotype (first isolate)	Additional serotype detected by cloning
BD75	AAV3B 98%	AAV3B 96%
BD117	AAV5, 8	AAV2
BD125	AAV2	AAV6 98%
IS8	AAV2	AAV2
IS12	AAV2 97%	AAV2 100%
IS15	AAV8 96%	AAV2 99%
1S17	AAV2	-
IS20	AAV2	AAV8 99%
IS21	AAV2 93%	AAV3B 99%
IS46	AAV2	AAV2

Please note that the percentages listed here denote the percentages of sequence identity with the most identical serotype. Unless otherwise indicated, the sequence analysed shares 100% sequence identity (homology), with the most identical AAV serotype. BD denotes blood donor and IS denotes immunosuppressed patient. When the same serotype detected by sequencing of purified PCR products was the sole serotype (with 100% homology) found in analysed clones, it is typed in black. If the same serotype was detected again, but with sequence identity less than 99%, it is typed in green. If a new serotype was detected, it is typed in blue.

4.5 Amplification of the Entire Capsid-coding Region.

In some AAV-positive blood donors' samples, the sequenced isolate had ≤ 98% homology with known human AAV serotypes. To get more information and possibly discover new serotypes, cap PCR was developed. The cap1 primers match serotypes AAV1- 3, 6- 8 and 10 completely, while there are two mismatches for AAV11 and 12 in the reverse primer. Cap2 primers match six serotypes; AAV1-3 and 6-8. Non-template control (nuclease-free water) and total genomic DNA from HeLa cells were used as negative controls, while pTAV-2.0 was used as positive control and to determine the sensitivity of PCR. Using *cap*1 and *cap*2/*cap*2_new primers, amplification of the entire capsid-coding region was carried out for six blood donors' samples, whose sequences share < 98% sequence similarity with known wild-type AAVs (Figure 4.16).

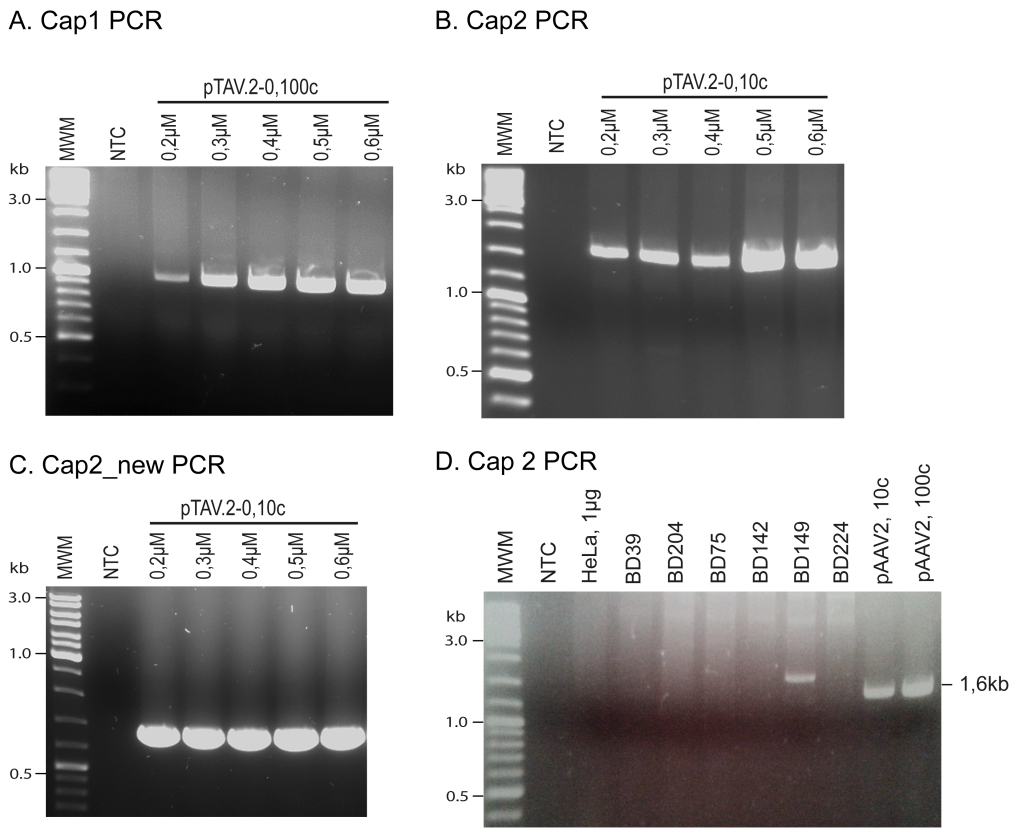


Figure 4.16 Cap PCR.

A. PCR was carried out using *cap*1 primers and testing different primer concentrations. **B.** PCR was carried out using *cap*2 primers and testing different primer concentrations. **C.** Representative gel photo of PCR *cap*2_new primers and testing different primer concentrations. D. PCR was carried out using *cap*2 primers on different blood samples. Primer concentration 0.5 µM was used for all *cap* primer sets.

The *cap* region of one sample was successfully amplified. The PCR product was cloned and sent for sequencing (BD149, AAV1 98%)[1]. Two sequences could be identified in this sample (AAV1 99% and AAV1 93%), consistent with early evidence elucidating molecular evolution in AAV (269).

[1] Cloning of purified PCR product of this sample and submission to sequencing was done by Melanie Heßler and Dr Daniela Hüser.

4.6 Analysis of PBMCs for the Presence of AAV Helper Viruses.

The high prevalence of AAV-DNA in PBMCs of blood donors significantly increasing -almost doubling- in immunosuppressed patients strongly supports the notion of AAV reactivation in immunosuppressed patients as a consequence of reactivation of any of its helper viruses. These include HCMV, EBV and HHV6, which are known to be reactivated and cause disease under immunosuppression, as described above. We investigated a possible correlation with the described AAV helper viruses known to persist latently in PBMC subpopulations. About 250 ng of some AAV-positive blood donors and immunosuppressed patients DNA was PCR analysed for HCMV, HHV6, and EBV DNA by our collaborator Dr Lassner, IKDT Berlin (with new numeration to exclude bias). Our analysis of the PCR results revealed the absence of HCMV DNA in all of the examined leukocyte samples of AAV-positive blood donors, (n= 46), which seems normal to healthy donors. HHV6 was detected at a low rate (7%, n= 71). In AAV-positive immunosuppressed patients compared to blood donors, the detection rate of HCMV DNA significantly increased (14%, n= 28, $p < 0.02$). The HHV6 detection rate was also higher in immunosuppressed patients (14%, n= 28), but did not reach statistical significance. Of five HCMV-positive immunosuppressed patient samples, three simultaneously had HHV6, but none had EBV. Surprisingly, EBV prevalence was higher in blood donors than immunosuppressed patients (33 %, n= 46, vs. 20 %, n= 25) (Figure 4.17).

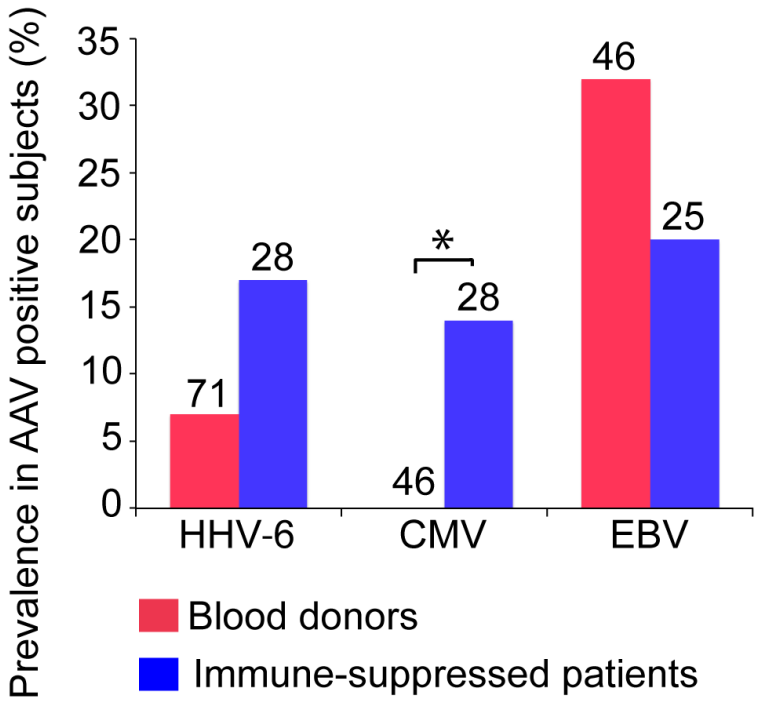


Figure 4.17 Screening for AAV Helper viruses in some AAV-positive blood donors and immunosuppressed patients.

Leukocytes DNA from some blood donors and immunosuppressed patients were tested for the co-presence of HHV-6, CMV, or EBV-DNA by IKDT using previously described nested PCR (270). The number of samples tested is indicated at the top of each bar. An asterisk "*" denotes a statistically significant difference ($p < 0.02$), calculated using Fisher Exact test.

4.7 Persistence of AAV in $CD3^+$ T-Lymphocytes

The high prevalence of AAV-DNA in human PBMCs observed in this study suggests that PBMCs may be one of the preferred sites for AAV persistence. It was interesting to identify the target leukocyte subpopulation/s for AAV persistence. To investigate this hypothesis, leukocytes were separated into $CD14^+$, $CD3^+$, and $CD14^-/CD3^-$ cell fractions by two successive rounds of immunomagnetic bead selection, using CD14 and CD3 microbeads, in addition to LS columns, or into $CD3^+$ and $CD3^-$ cell fractions, by immunomagnetic bead selection, using CD3 microbeads and LS columns (Figure 4.18). To do this, PBMCs were purified from 50 ml freshly drawn blood samples of five previously tested AAV-positive, healthy blood donors. Then, PBMCs were separated by magnetic-activated cell sorting (MACS) into different fractions, as stated above. DNA was extracted from different fractions and analysed by AAV PCR assay.

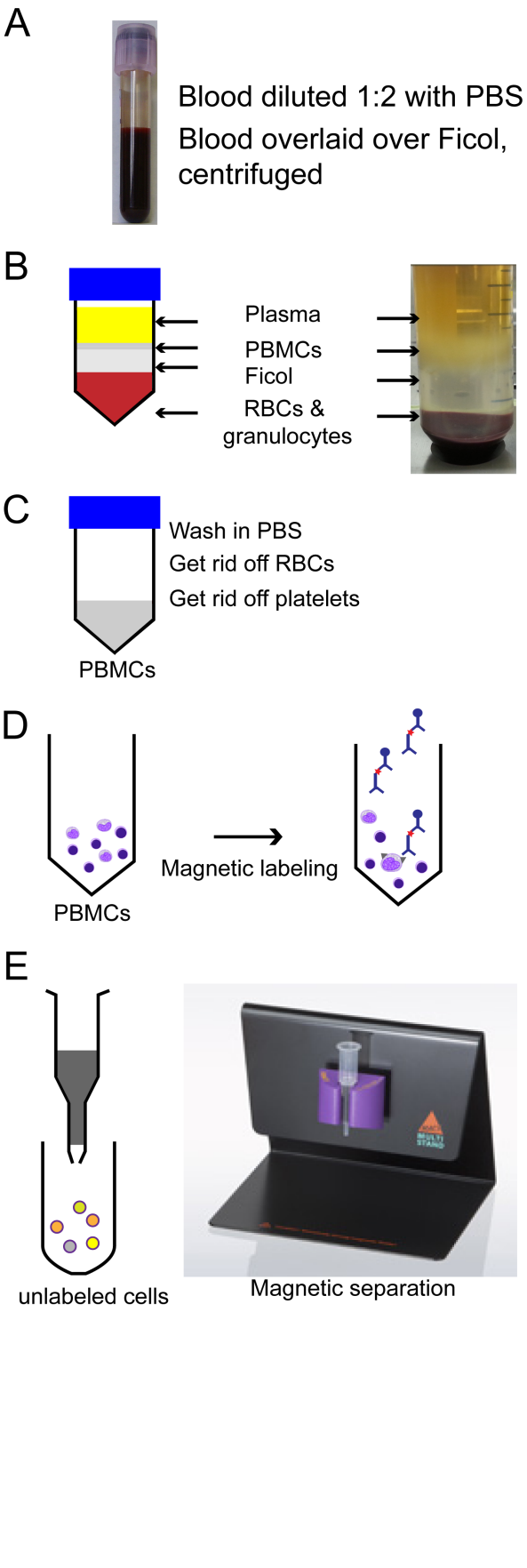
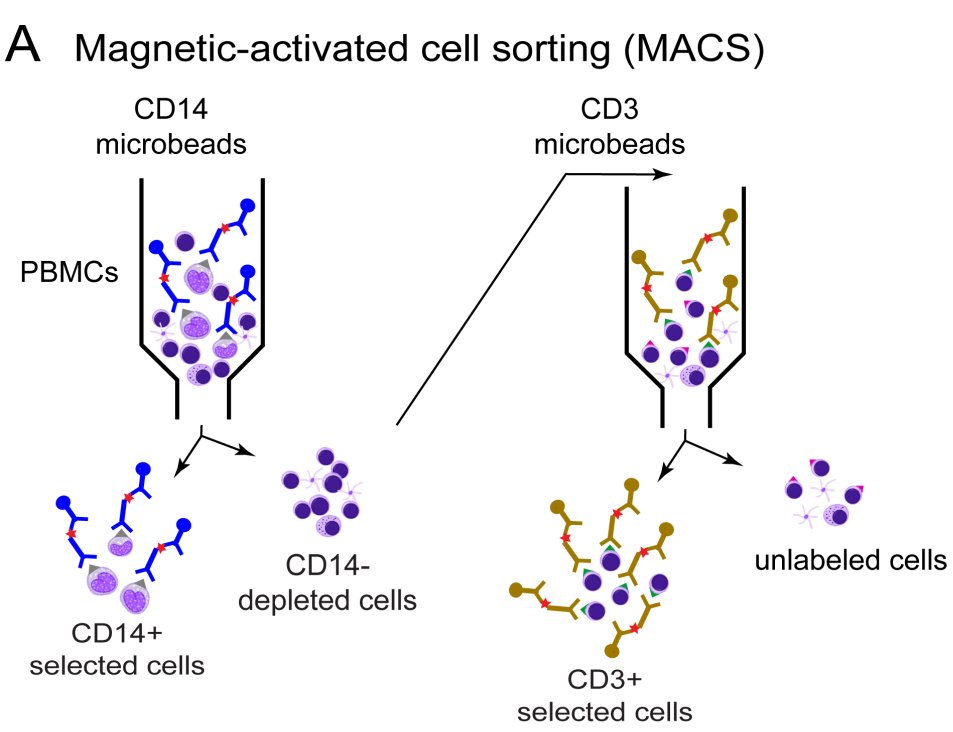

A
Blood diluted 1:2 with PBS
Blood overlaid over Ficol, centrifuged
B
Plasma
PBMCs
Ficol
RBCs & granulocytes
C
Wash in PBS
Get rid off RBCs
Get rid off platelets
PBMCs
D
PBMCs
Magnetic labeling
E
unlabeled cells
Magnetic separation
labeled cells
Positive selection

Figure 4.18 Magnetic-activated cell separation (MACS) protocol.

A. and **B.** Ficoll density gradient separation of peripheral blood mononuclear cells (PBMCs) from freshly drawn blood. **C.** PBMCs were washed in PBS and purified from remnant contaminating RBCs and platelets. **D.** PBMCs were magnetically labelled using the corresponding microbeads, CD14 or CD3 microbeads. **E.** Magnetic separation by positive selection was carried out, where the unlabelled cells pass through the column applied to the MACS adaptor, while the labelled cells were gained by applying a plunger to the column away from the magnetic field, i.e. the MACS adaptor. For more details, please refer to the methods section. All elements of the figure displayed are original except for MACS separator image which was downloaded from Miltenyi Biotec website at the following URL: http://www.miltenyibiotec.com/en/products-and-services/macs-cell-separation/manual-cell-separation/separators/midimacs-separator-and-starting-kits.aspx (271)

Purified PCR products from positive samples were submitted to DNA sequence analysis, to confirm specificity and identify the concerned AAV serotype. In four samples, AAV2 DNA was detected exclusively in the $CD3^+$ fraction (Figure 4.19, B and C, samples BD15, 132, 244 and 258). In sample BD258, AAV5 DNA was detected as well, remarkably, in both the $CD3^+$ and $CD3^-$ fractions (Figure 4.19, D). Sequence analysis of 1st round and nested PCR products showed that the $CD3^-$ fraction solely contained AAV5 DNA. The $CD3^+$ fraction contained both AAV2 and AAV5 DNA, selectively amplified by nested PCRs, while sequence analysis of 1st round PCR product of $CD3^+$ fraction revealed the presence of AAV2 DNA. Sample BD104 was negative for AAV, although it had tested positive twice (24 months and 2 months before) (Figure 4.13). PCR was repeated for this sample (BD104) with all controls, and the result was confirmed. None of the $CD3^+$ or $CD3^-$ fractions contained HCMV or HHV6 DNA[2].

[2] HCMV and HHV6 PCR was carried out on 250 ng DNA from different leukocytes fractions by Dr Dirk Lassner, IKDT.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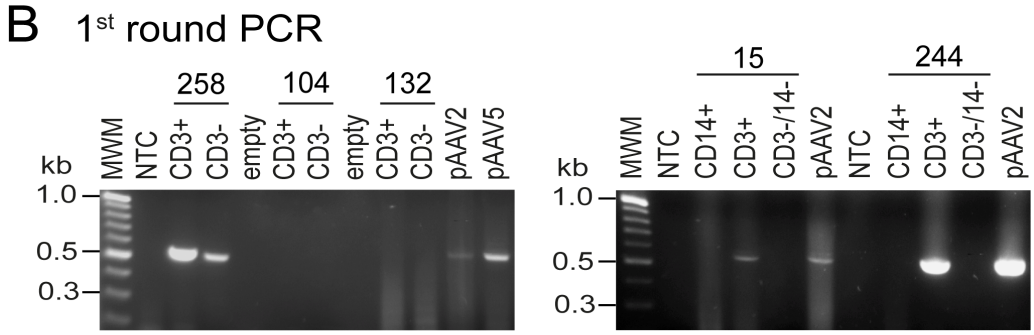
CD3 microbeads
PBMCs
CD14+ selected cells
CD14- depleted cells
CD3+ selected cells
unlabeled cells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0.5
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MWM
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104
132
CD3+
CD3-
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pAAV2
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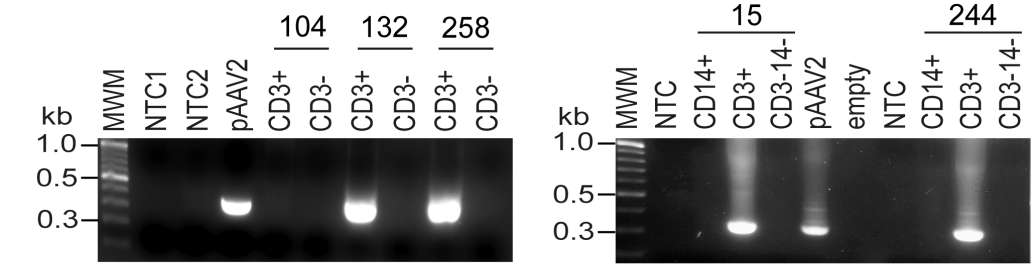
15
244
CD14+
CD3-/14-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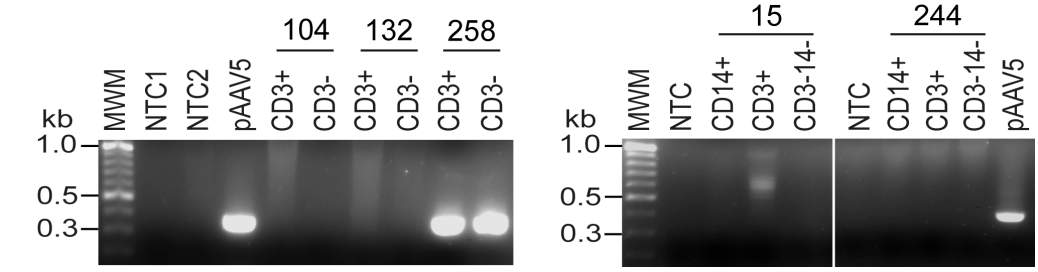
D AAV5 nested PCR
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CD3+
CD3-
15
244
CD14+
CD3-14-
NTC
pAAV5

Figure 4.19 Screening of some leukocytes' subpopulations for AAV persistence.

A. Schematic representation of MACS as performed in this study. Peripheral blood mononuclear cells (PBMCs) were separated using CD3, and CD14 microbeads. Five previously tested, AAV-positive blood donors were tested. Two samples (BD15, BD244) were separated into $CD14^+$, $CD3^+$, and $CD3^-/14^-$ fractions. Three samples (BD132, BD104, BD258) were separated into $CD3^+$ and $CD3^-$ fractions. **B.** 1st round PCR on different leukocytes fractions for five previously tested AAV-positive blood samples, to test the presence of AAV-DNA. Samples BD15, BD132, and BD244 showed signals only in the $CD3^+$ fraction, while sample BD258 showed signals in both $CD3^+$ and $CD3^-$ fractions. **C.** Nested PCR with AAV sero nested primers, showed the presence of an AAV-specific band exclusively in the $CD3^+$ fraction of four samples (BD15, BD132, BD244, and BD258). **D.** AAV5 nested PCR using AAV5 nested primers showing the presence of an AAV5-specific band in both $CD3^+$ and $CD3^-$ fractions of sample BD258. Sequencing of 1st round and nested PCRs products confirmed the presence of AAV2 selectively in $CD3^+$, while AAV5 was found in both $CD3^+$ and $CD3^-$ fractions. This figure was previously published in Hüser, Khalid et al. J Virol 2016, Dec 7. pii: JVI.02137-16

4.8 Investigating the Biologic Activity of AAV Genomes, and Rescue of Infectious AAV from AAV-Positive $CD3^+$ Fraction.

After observing the high prevalence of AAV-DNA in blood donors, a prevalence that significantly increased in immunosuppressed patients, it seemed important to investigate the presence of biologically active intact AAV genomes in these PBMCs, which are infectious in a secondary infectious assay, i.e. AAV rescue upon supplying the helper functions needed for its replication (40, 194, 82). We wanted to evaluate the ability of AAV genomes to undergo active DNA replication in the presence of a helper virus, such as adenovirus or herpes virus, and produce progeny virions that are infectious in a secondary infection assay. To assess this, a pilot experiment was carried out to infect $CD14^+$ and $CD14^-$ cells from an AAV-positive donor with HSV-1 in the first round of infection. Virus DNA extracted by modified Hirt extraction was quantified by PCR, but no significant difference in the amplification efficiency between different sets was noticed. The second round of infection was done using freeze-thaw lysate from the first round of infection with or without AD-2 to infect different sets of HeLa cells. No significant difference in AAV titer quantified by qPCR was noticed between various sets. Background controls included HeLa cells infected with freeze-thaw lysate alone or Ad-2 alone or non-infected at all. The copy number of the virus detected by qPCR was extremely low, which might explain the inability to detect a noticeable difference in AAV replication

efficiency between different sets. Because of that, in addition to the inconvenience of obtaining 50 ml fresh blood from a significant number of previously tested AAV-positive volunteers, no further optimization of this method was carried out. Instead, colleagues Dr Hüser, Dr Weger and Mrs Hammer used transfection technique to investigate the reactivation of AAV upon infection with helper virus. Shortly, genomic DNA from previously tested AAV-positive blood donors' or immunosuppressed patients' samples were used to transfect HEK293 cells with and without helper plasmid (to provide adenoviral helper functions). In about 60% of the tested samples, AAV replication was higher in AAV-positive samples transfected with a helper plasmid. Negative controls used were three previously tested AAV-negative blood donors DNA. For more details, please refer to Hüser, Khalid et al. Journal of Virology 2016, Dec 7. pii: JVI.02137-16.

5 Discussion

AAV is attracting more interest and wide acceptance as a promising gene therapy vector due to its broad tissue tropism, relative safety, ability to transfect even non-dividing cells, and long-term expression of the transgene (181). However, a major unresolved challenge for the success of AAV-mediated gene therapy is the presence of pre-existing antibodies, or the development of AAV antibodies upon administration of the first dose of rAAV vector, limiting the effectiveness and restricting administration of further doses, if needed (234). Despite the ubiquity of AAV in diverse species and tissues (130, 84, 272), most of our knowledge about AAV is, in fact, drawn from *in vitro* experiments, and little is known about AAV *in vivo* biology, making the struggle to optimize AAV-mediated gene therapy vector even harder. AAV ubiquity and high anti-AAV seroprevalence (136, 141, 148, 146, 144, 273, 150) indicate either that antibodies are not protective as supposed before (274), so that reinfection with circulating virus in the community might occur frequently, or that AAV may remain latent in infected tissue (1, 92, 82, 275), which sounds plausible in view of its biphasic life cycle. Nonetheless, the target cell type for AAV *in vivo* persistence has not yet been identified.

Our main focus of the study was to detect target cells for AAV persistence to approach a better understanding of human *in vivo* biology of AAV. As latent virus does not replicate, the copy number of integrated/episomal persistent AAV in healthy blood donors was expected to be relatively low. Optimizing PCR sensitivity was of supreme importance. So our first goal was to establish a highly sensitive and specific PCR assay that allows detection of the broad spectrum of human AAVs known to date, then to apply that assay to screen leukocytes to test our hypothesis of AAV persistence in these cells.

Our postulation that AAV might persist in leukocytes was based on previous findings, including wide distribution of AAV in different tissues in human, especially bone marrow, liver and spleen (130). Besides, blood cells, i.e. leukocytes are common cells that are readily distributed in all tissues. AAV was also more promptly detected in PBMCs in a subset of NHP after iatrogenic infection with AAV (83).

In this work, we report a high prevalence of AAV-DNA in healthy blood donor leukocytes, with serotype distribution consistent with reported seroprevalences (148, 146, 144, 150). This finding, when compared to the rather sporadic detection of AAV-DNA in other cells (130, 84), suggests leukocytes as a preferential site for AAV persistence. However, further studies using our PCR assay or a comparably sensitive PCR and screening different tissues are needed to

confirm or relinquish our notion.

The significantly higher prevalence of AAV in immunosuppressed patients is strongly suggestive of reactivation of persistent AAV infection, mostly as a consequence of reactivation of associated AAV helper virus infection. Herpesviruses and adenovirus are known to reactivate upon immunosuppression, often with adverse clinical outcomes (112, 276-279). Though we did not observe any significant correlation between the higher prevalence of AAV in immunosuppressed patients and the associated increase in co-prevalence of its helper viruses tested in this study (EBV, CMV, HHV-6), their mutual interactions and the potential impact on *in vivo* pathogenesis are challenging and far too complex and require further studies. Finally, the increasing application of AAV-based vectors in gene therapy calls for greater insight into the natural course of wild-type AAV infection in order to further evaluate the safety of AAV vectors for clinical use.

5.1 Biology and Transmission of AAV

AAV-DNA was isolated from tonsils and adenoids of children, albeit at a relatively low rate (7%) (84), and thought to be transmitted along with adenovirus infection, as seroepidemiology closely follows that of adenovirus (141, 149). However, AAV seroprevalence steadily and significantly increases with age, suggesting that AAV infection might start in childhood, but reach a peak later in life (148, 280, 149, 281, 147). It seems that AAV may be transmitted by the respiratory route, and might replicate initially at the primary site (i.e. adenoid or tonsils). From there, AAV may disseminate by haematogenous spread (as a free virus or blood cell-borne, i.e. in leukocytes). Supporting this postulation is the observation that AAV was distributed in many human tissues, particularly bone marrow, liver and spleen (130). The most plausible explanation is that AAV resides in blood cells and reaches almost all tissues via blood.

Based on high AAV seroprevalence and AAV-DNA isolation from different tissues, especially from leukocytes, we suggest that AAV remains latent in target cell/s (i.e. leukocytes) till reactivated by a helper virus, rather than the frequent occurrence or recurrence of AAV infections.

5.2 Prevalence of AAV in Healthy Blood Donors

Rapid progress is being made in AAV-mediated gene therapy, whereas the gap between the extensive *in vitro*-study-based information about wild-type AAV and AAV vectors, and the very limited knowledge about wild-type AAV *in vivo* biology is still widening. Reported AAV seroprevalence ranges from 30-90% (282, 136, 141, 283, 143, 148, 280, 146, 144, 149, 145, 150, 147, 284), yet the target cell/s for AAV replication and persistence has/have not been identified, and AAV-DNA has only rather been detected sporadically in different human tissues and samples (261, 151, 162, 130, 84).

We observed a high prevalence of AAV-DNA in healthy blood donors' leukocytes (34%), leading us to consider leukocytes as a potential *in vivo* site for AAV persistence. Moreover, despite using consensus primers, the percentage of mixed infection was unexpectedly high (~11%) (Figure 4.11). This is consistent with AAV seroprevalence studies reporting between 50 and 100% co-prevalence of IgG antibodies to capsid proteins of two different AAV serotypes (144), and 40% and 70% seropositivity against each tested AAV serotype (AAV2, 3, 8, and AAVLK03) at neutralization titre 1/80 and 1/20, respectively (150). This co-prevalence is highly suggestive of mixed infection, cross-reaction, or both.

The distribution of different serotypes we observed was, to a great extent, proportional to the reported seroprevalence. AAV2 DNA was the most frequently encountered serotype in our study (77%), followed by AAV5 (19%). This correlates with many studies that have found anti-AAV2 IgG or neutralizing antibodies (NA) to be the most prevalent AAV antibodies (146, 144, 145, 150). Anti-AAV5 IgG or NA were generally lower, which was presumed to be at least partially due to cross-reaction, as most of the anti-AAV5-positive subjects were also positive for AAV2 with even a higher titre against AAV2 (144, 145). Though our findings support the notion that mixed infection is much more likely than previously anticipated, cross-reaction or previous exposure may still account for part of the seroprevalence proportion.

We isolated AAV1 and 6 DNA, though less frequently than expected (4% and 6%). Most seroprevalence studies reported anti-AAV1 antibodies to be the second most prevalent antibodies, with a prevalence comparable to AAV2 (146, 144, 147), whereas the prevalence of anti-AAV6 antibodies ranged from 20 to 40% (280, 144). Cross-reaction is believed to account for at least a considerable proportion of anti-AAV1 seroprevalence, especially in light of the observation that all anti-AAV-1-positive sera were also positive for anti-AAV2 NA (144, 147), and in almost all anti-AAV-1 NA-positive sera, the anti-AAV2 NA titre was

regularly higher (147). In fact, the simultaneous presence of antibodies/neutralizing factors against more than one AAV serotype was found in a considerable percentage of AAV-positive sera (280, 150).

We detected AAV3B DNA only once (1%) in AAV-positive blood donors' leukocytes, whereas AAV8 DNA was detected in four samples (5%), three of which were mixed with other serotypes (Table 4.1). Few studies have addressed anti-AAV3 antibody prevalence (285, 141, 150). While one recent study reported anti-AAV3 neutralizing antibodies (NA) prevalence of about 40 and 50% (in healthy donors and inflammatory bowel disease (IBD) patients, respectively), and less than 20% of them having a high titre (281), another more recent study reported AAV3 seroprevalence comparable to that of AAV2 (89% vs. 92%) (150). This difference can be attributed to differences in geographical distribution, as both studies were restricted to certain populations. It should be noted that the sequence homology between AAV2 and 3 exceeds 83% (40, 125, 3), so cross-reacting antibodies might be responsible for that relatively high inconsistent seroprevalence. One caveat must be stressed, namely that the consensus primers we used have four mismatches for AAV3 and AAV9, which might have led to an underestimation of these serotypes. For AAV8, however, in most studies anti-AAV8 antibodies were the least prevalent of all serotypes, making it a favourable candidate for gene therapy (146, 144, 281, 145). Our data correlates with the previous findings of Gao and colleagues, where they detected AAV isolates dispersed in different tissues of human (19%) and non-human primates (18%) (130). They classified AAV2 and homologous isolates into a new clade "B", which was the most prevalent clade detected in human, followed by clade C, composed of AAV2/3 hybrids, then clade E (AAV8). Clade A (AAV1/6) and clade F (AAV9) were identified rather less frequently (5%) (Figure 1.6) (130). In the present study, we did not detect AAV7, 11, 12 or 13 in any of our samples, confirming the previous assumption of their non-human origin (126, 117, 130-133). However, it should be noticed that the nested primers might not hybridize efficiently with AAV11 or -12, due to the mismatches in the reverse primer (Figure 4.1). The primers we used, especially the nested primers, also have many mismatches for AAV4, so that its prevalence in human cannot be estimated in this study. Therefore, the previous anticipation that it is a non-human serotype cannot be further confirmed or rejected. Again, by using consensus primers, some AAV serotypes might be underrepresented/underestimated in the case of mixed infection with more than one AAV serotype, where one serotype might be found in higher number, or privileged in binding the primers than the other, and therefore might be amplified more efficiently. Our AAV PCR assay may still not be sensitive enough to appreciate the actual prevalence of

AAV-DNA and the distribution of the full spectrum of human serotypes, as indicated by the apparent gap between some AAV serotypes' low detection rates and their relatively higher seroprevalence. The higher percentage of different AAV serotypes infection, as indicated by their seroprevalence, than their rate of persistence could be explained by either the ability of the immune system in some cases to get rid of AAV infection, while anti-AAV antibodies last lifelong, whereas in other cases AAV remains quiescent (persists). Another possibility is that cross-reaction might account for the overestimation of the seroprevalence of some AAV serotypes.

Most AAV sequences we isolated were largely conserved, as the amplicon locates in a relatively conserved region of the *cap* gene. The region of isolated sequences coding for phospholipase A2 (PLA2) is highly conserved, which seems logical as it is essential for virion infectivity (61). Only a few sequences showed mutations from the most identical serotype. Interpretation of the potential significance of these mutations revealed that all the mutations in these sequences lie downstream from the phospholipase-coding region of the *cap* gene.

5.3 AAV Prevalence in Immunosuppressed Patients

One major barrier to the success of AAV-mediated gene therapy is the preponderance of pre-existing neutralizing antibodies (280, 146, 144, 281, 286, 147), or the development of Nab after first vector administration hindering transgene expression (236, 287, 220, 234). Transient immunosuppression (IS) or immune-modulation has been used to evade the immune response and allow long-term expression of the transgene (236, 220, 288). However, in some recent studies, the effect of immunosuppression on AAV immune response was contrary to these expectations. In a study carried out on transplant patients before and after immunosuppressive therapy, it was observed that the numbers of AAV capsid-specific $CD4^+$ and $CD8^+$ cells were higher in transplant patients at baseline and also in comorbid controls compared to age-matched healthy adults, though the average total number of T-cells was reduced or the same (237). In transplant patients, $CD4^+$ effector memory T cells were more common before IS (31%) than in control cohorts. These cells even increased after initiation of IS (51%) and underwent functional changes, raising questions about the efficacy of immunosuppression in modulating immune response to AAV gene therapy. Central memory $CD4^+$ and $CD8^+$ cells were also more frequent in transplant patients than in healthy donors. The average number of AAV capsid-specific $CD8^+$ central memory cells even increased after initiation of IS, while the total number of AAV capsid-specific $CD8^+$ cells did not change and

the total number of AAV capsid-specific $CD4^+$ cells decreased (237). The most acceptable explanation, as suggested by the researchers of this study, is a possible reactivation of persisting AAV, since some similar changes were observed for comorbid cohorts, while no change in antibody titre was noticed between the three cohorts, minimizing the presumption of acquiring new AAV infection upon immunosuppression (237). Another study addressed the features of AAV capsid-specific T-cells in humans and non-human primates (NHP) to clarify the cause of lower or shorter transduction of AAV vectors in humans than in NHP, though the latter have a higher frequency of AAV capsid-specific T-cells. They observed that not only the frequency of the AAV capsid-specific T-cells differed, but also their response, activity, differentiation, and subset distribution, where memory cells exceeded effector cells in human, and vice-versa in NHP, for different T-cells subsets, i.e. $CD4^+$ and $CD8^+$. Additionally, no evidence of T-cell exhaustion was observed in AAV capsid-specific T-cells or non- AAV capsid-specific antigen-experienced T-cells in either species (289). Our interpretation is that these findings suggest reactivation of a persistent AAV infection upon immunosuppression, where AAV persist in leukocytes in humans, but not in NHP. Affirming our notion are the previous findings of Gao and colleagues (130), where the distribution of AAV-DNA in various tissues was different between human and non-human primates. AAV-DNA was more frequently detected in bone marrow, liver (~30%) and spleen (~18%), but not in heart tissues, and blood was not tested. Whereas in non-human primates, AAV-DNA was more readily isolated from lymph nodes and liver (~50%), followed by spleen and heart tissues (~40%), but rather rarely in bone marrow and blood (~5%). The AAV prevalence was almost the same in human and NHP (19% and 18%, respectively (130). This would suggest a different AAV persistence pattern between human and non-human primates.

We observed a significantly higher prevalence of AAV-DNA in immunosuppressed patients (76% vs. 34%, $p < 0.000001$) (Figure 4.11, D). The percentage of mixed infection also increased significantly (45% vs. 11%, $p < 0.0001$) (Figure 4.11, E). Nevertheless, the distribution of AAV serotypes in immunosuppressed patients followed almost the same pattern as in blood donors. AAV2 was the most frequently detected serotype (90%), followed by AAV5 (39%). Other serotypes AAV3B (6%), AAV8 (10%), and AAV9 (3%) were also detected (Figure 4.12). Intriguingly, AAV1 and -6 were not detected in any immunosuppressed patient samples. A negative correlation was also noticed reciprocally between AAV1 and -8 seropositivities (146), which correlates with our findings in immunosuppressed patients (AAV1 was not detected at all, whereas AAV8 was detected three times (10%)). However, the sample number of the immunosuppressed patients we

screened is not big enough to extrapolate any conclusion or confirm this correlation. Also, a significantly lower prevalence of neutralizing antibodies against AAV1, -5, and -8 in patients with inflammatory bowel diseases (IBD) than in healthy controls was reported, while the opposite was observed for AAV6 (281). However, the lower prevalence of NA against these AAV serotypes could be attributed to the described weaker humoral immune response in IBD patients (290). More information is still sought to clarify the *in vivo* immune response and natural history of AAV infection.

We isolated AAV9 once in an immunosuppressed patient, a serotype first isolated from three out of 259 human tissue samples (130). In a recent report, 70% of cytokine-primed $CD34^+$ peripheral blood stem cell (PBSC) samples were found to be AAV positive, where all isolated sequences were wild-type AAV9 or shared highest sequence identity with AAV9 (291). An explanation for the rather high detection rate of an otherwise sporadically detected serotype might be a selective tropism of AAV9 for $CD34^+$ cells or a better binding capacity of the primers used in this study with AAV9.

Mixed infection was significantly higher in immunosuppressed patients compared to healthy blood donors (45% vs. 11%). Follow-up studies with sets of AAV serotype-specific primers will be needed to further evaluate the unexpectedly high frequency of mixed infections of varying serotypes. The higher proportion of mixed infection in immunosuppressed patients gives more support to our postulation that reactivation of AAV helper virus might result in reactivation of persistent AAV infection in immunosuppressed patients, which seems like a possible explanation for the significantly higher prevalence of AAV in immunosuppressed patients than in blood donors. However, more evidence is needed to support this notion; like rescue of the virus *in vitro* upon co-infection of the leukocytes with a helper virus, or a higher detection rate of any of AAV helper viruses in immunosuppressed patients than in blood donors.

Most AAV helper viruses, including human herpes viruses are reactivated in immunocompromised patients, often with worse prognosis (112-115) and may thereby lead to AAV replication. However, we observed only a modestly significant increase in CMV detection rate in AAV-positive immunosuppressed patients than in AAV-positive blood donors. HHV-6 detection rate was higher in AAV-positive immunosuppressed patients than in blood donors, although the difference in detection rates did not reach statistical significance. The mutual *in vivo* interaction of AAV and its helper viruses, and whether and how AAV reactivation influences clinical course and outcome of a co-infecting helper virus, mandate

further studies. Besides, the probability that the two virus groups meet *in vivo* is not so high, considering the low AAV frequency of 1 ssDNA genome in 2×10^5 PBMCs of healthy individuals. Immunosuppression, encountered as transient episodes during diverse disease states, increases the probability of AAV activation, possibly by an altered T-cell response (237) or by herpesvirus coinfection, as we hypothesize, or both.

Nonetheless, we observed a non-significant higher detection rate of EBV in blood donors (32%) than in immunosuppressed patients (20%). EBV is reactivated in immunocompromised patients (292) and has a different latency pattern in B-cells than in immunocompetent hosts (293). As peripheral blood mononuclear cells have been considered non-desirable specimens for diagnosis or prognosis of many EBV-associated diseases in immunosuppressed patients (293), and we screened DNA extracted from these cells for the presence of EBV, that might clarify the unexpected lower prevalence of EBV in immunosuppressed patients than in healthy blood donors.

5.4 Fluctuating AAV Detection During Long-Term Persistence

Repeated detection of AAV-DNA in some positive blood donors over a time interval of two years gives more evidence to the hypothesis of AAV persistence in leukocytes (Figure 4.13). An interesting finding is that one AAV-positive blood donor (BD132) tested negative after nine months of being positive at first examination, and then was shown to be positive again after another 14 and 15 months (Figure 4.13). This supports our hypothesis that AAV persists in leukocytes, and it seems that the virus load fluctuates and may at some time points be under the sensitivity detection limit of PCR. Our PCR assay detects 1 to 10 AAV copies in 1µg genomic DNA (~ 10^5 diploid cells). When the AAV level drops below this limit, AAV-DNA will no longer be detectable.

It is worth noting is that the serotype/s detected for the same donor was/were not always the same one/s detected repeatedly (Table 4.3). This finding may reflect a fluctuation in the virus load in the case of infections with more than one AAV serotype, which are then detected alternately. In other words, in the case of mixed infection, different serotypes could be detected at various time points, simultaneously or alternately.

The high prevalence of neutralizing antibodies against AAV serotypes (144), not variable among different cohorts including healthy donors, transplant patients, and comorbid cohorts, in spite of the significantly higher prevalence of AAV capsid-specific T-cells in transplant patients and comorbid cohorts (237), suggests persistent infection as a more convincing

explanation. Anti-AAV2-IgG seroprevalence was increased in pregnant women compared to controls, without a corresponding increase in IgM, which suggests reactivation of latent infection due o immunotolerance associated with pregnancy (148).

Another remote possibility is that AAV circulates in the community and recurrent infection often occurs, as the antibodies did not appear to play a protective role (274). Also, in support of this consideration is the relatively frequent detection of anti-AAV2 IgM antibodies after age 30 and in pregnant women (148). This could be correlated more with the significant age-related increase in AAV seroprevalence, though (148, 281, 147), which means that new infections occur steadily, adding new members to the ubiquitous AAV human hosts. Immune tolerance in pregnancy might increase the predisposition to new AAV infection. Repeated detection of varying AAV serotypes during follow-up of individual subjects could, therefore, be interpreted as fluctuation of viral load during persistent infection with different AAV serotypes. Fluctuation of viral load during the course of persistent and chronic infection was described for many viruses (294-297). Supporting that postulation is the significantly higher frequency of mixed infection we observed in immunosuppressed patients compared to healthy donors, implying reactivation of persistent coexisting serotypes. Also, corroborating that assumption is the retrieval of missed AAV serotypes upon cloning of some PCR products from AAV-positive hosts whose raw data sequences showed underlying sequences (Table 4.4).

5.5 T-Lymphocytes as Sites of AAV Persistence

In the present study, we observed that AAV2, the most prevalent serotype in PBMCs, exclusively resides in $CD3^+$ T-lymphocytes. This novel finding is consistent with the previous detection of AAV in many human, and non-human primate tissues, particularly the bone marrow, lymph nodes, liver and spleen (130), as lymphocytes are pervasive cells found in almost every tissue. In a recent study, AAV capsid-specific T-cells were frequently detected (~50%) in humans and characterized by predominant memory cell response. In contrast, in NHP tested in the same study, AAV capsid-specific T-cells were more frequent and mainly effector cells, and differed in their function. Nevertheless, the duration of AAV-mediated gene transfer was shorter in human than in NHP (289). Exhaustion and decreased functions of T-cells upon recurrent activation associated with continuous production of AAV proteins due to the ubiquity of adenovirus in NHP is an explanation the authors offered. However, when correlated with the rather rare detection of AAV in blood and bone marrow of NHP, in

comparison to the high prevalence of AAV in human bone marrow (130), our assumption of AAV persistence in human T-cells seems to be a more conceivable explanation of the stronger T-cell immune response in human compared to NHP. AAV *in vivo* life cycle is presumably different in the two species. Pre-existing T-cells to AAV antigens generated during natural infection could lead to cessation of transgene expression in human subjects who need AAV-mediated gene therapy (226, 298). A thorough investigation is required to determine whether the persistence of AAV-DNA elicits and maintains a high level of specific cell-mediated immune response, and if it may modulate or boost the immune response. This accentuates the importance of screening not only for neutralizing antibodies in AAV gene therapy recipients, but also for AAV-DNA, which might denote persistent AAV infection .

The high AAV seroprevalence of up to 80 to 90% (148, 146, 144, 147) compared to the lower prevalence of AAV capsid-specific T-cells (~50%) (289), and AAV-DNA prevalence in our study (34% and 76% in blood donors, and immunosuppressed patients, respectively), when taken together could suggest the following scenario: AAV is circulating in the community, and many people acquire asymptomatic AAV infection. In such a case, even if the virus is more or less cleared up by the immune system, the infected people develop antibodies against AAV lifelong. A lower percentage might still harbour AAV in a latent stage (mainly in leukocytes as we suppose or may be in other sites as well), which could be reactivated later on, upon immunosuppression.

The frequency of AAV capsid-specific T-cells reported in transplant patients compared to healthy controls and comorbid cohorts was higher, whereas AAV-neutralizing antibody titres in the three groups and in different samples withdrawn four weeks apart remained almost unchanged (237). This strongly advocates the important role we assumed for immunosuppression in AAV reactivation. The increased AAV-DNA detection rate in PBMCs of immunosuppressed patients we observed further substantiates the claim that leukocytes are a preferential site for AAV persistence.

Furthermore, human herpesviruses are regularly activated upon immunosuppression and may thereby lead to AAV replication. We thought that HCMV could have been a potential candidate; however, it had not been shown to persist in T-lymphocytes (30). Persistence in T-lymphocytes is known for HHV6 (299) which is highly prevalent in the human population with seropositivity rates of up to 90% (300). Interestingly, HHV6 carries a functional AAV *rep* gene homologue, which HHV6 had likely acquired during coinfection with AAV by non-homologous recombination (301). AAV *rep* homologues have also been acquired by

herpesviruses of other species, such as rat cytomegalovirus (302) and bat herpes virus (303). This may imply that some aspects of the mutual *in vivo* interaction between AAV and herpes viruses among species might be overlooked or undervalued.

In contrast to AAV2, which we detected exclusively in $CD3^+$ fraction, AAV5 was simultaneously present in both $CD3^+$ and $CD3^-$ fractions from the same donor. This may be interpreted as an indication of AAV5's variant cell tropism (128, 304). The very rare detection of AAV5 DNA in human tissues (124, 130, 84) further underlines its unique *in vivo* phenotype. AAV5 was also reported to have a different seroprevalence pattern and is supposed to be sexually transmitted (124, 8, 128). Follow-up studies with larger cohorts of AAV-positive subjects will help to corroborate or abandon our hypothesis.

The high preponderance of different AAV serotypes we observed in this study, mandates more studies to be conducted to get a better and closer understanding of the *in vivo* biology of this important gene therapy vector. Screening of different tissues for the presence of AAV using the sensitive PCR assay we developed, may help to discover other sites for AAV persistence. Investigating the *in vivo* molecular form of persistent AAV is vital to better explore the safety of rAAV in gene therapy. Recombinant AAV vectors (rAAV) are devoid of rep gene, and are shown to persist mainly as episomes (82, 214). However, if patients persistently infected with wild-type AAV are injected with a high dose of rAAV vectors, undesired recombination, as well as unwanted immune response might happen. This justifies the urgency for more projects addressing AAV *in vivo* biology in more details. We hope we could in this study pave the way somewhat for colleagues and scientists to get more insight into the AAV life cycle in humans. Careful surveillance before administration of rAAV-mediated gene therapy using our AAV PCR assay (or even to optimize a better one) might be needed to avoid unexpected side-effects.

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„Ich, Dina Khalid, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: [Prevalence and Characterization of AAV Persistent Infection in Leucocytes of Blood Donors and Immune-suppressed Patients] selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
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Ausführliche Anteilserklärung an der erfolgten Publikation

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# Equal contribution,

Beitrag im Einzelnen:
- Projektplanung und Protokollführung über die experimentellen Ergebnisse.
- Planen und Durchführen aller Experimente, die im Paper beschrieben sind, ausgenommen der Helfer Viren PCR und Transfektionsversuch (deren Ergebnisse in Abbildung (5) der Publikation gezeigt sind).
- Daten Zusammenfassung und Analyse.
- Erstellen des ersten Manuskriptversion, Anfertigung von Tabellen und Abbildungen (Abbildung 5 ausgenommen).
- Überarbeitung des Manuskripts und Tabellen und Abbildungen gemäß den Vorschlägen und Kommentaren von Prof. Dr. Heilbronn und Dr. Hüser.

Unterschrift, Datum und Stempel der betreuenden Hochschullehrerin

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Curriculum Vitae

For privacy reasons the curriculum vitae is not included in the electronic version.

Publication

- Poster Presentation, **Dina Khalid**.: High prevalence of adeno-associated virus (AAV) in human peripheral blood mononuclear cells indicative of T-lymphocytes as sites of AAV persistence, P276. 26th Annual Meeting of the Society of Virology, Münster, 6-9 April 2016.

- Hüser* D, **Khalid* D**, Lutter T, Hammer EM, Weger S, Heßler M, Kalus U, Tauchmann Y, Hensel-Wiegel K, Lassner D, and Heilbronn R.: High prevalence of infectious adeno-associated virus (AAV) in human peripheral blood mononuclear cells indicative of T lymphocytes as sites of AAV persistence. Journal of Virology 2016, Dec 7. pii: JVI.02137-16.

- **D.H. and D.K. equal contribution**.

The material and results in this thesis were previously published in one paper by the author (Hüser, Khalid et al. Journal of Virology 2016, Dec 7. pii: JVI.02137-16)i. Kindly notice that some phrases and paragraphs have been quoted verbatim or in part from this publication.

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Samuel Beckett