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Statement of the ZKBS on risk assessment of Adeno-associated viruses from primates and vectors derived therefrom

Adeno-associated viruses

Adeno-associated viruses (AAVs) are ubiquitously distributed in a variety of animals and humans, with a very narrow host range of individual serotypes [1; 2]. As a subgroup of the defective viruses (genus *Dependoparvovirus*), they belong to the family *Parvoviridae* [3].

AAV particles are not coated and are relatively stable in the environment even when dried out. The single-stranded DNA genome consists of the two open reading frames *rep* and *cap*. The four nonstructural proteins Rep78, Rep68, Rep52, and Rep40 encoded by *rep* are important for virus replication, expression of the structural proteins, and integration into the host cell genome. The three capsid proteins VP1, VP2 and VP3 are encoded by *cap* and form the icosahedral nucleocapsid. Hypervariable regions of the capsid proteins influence tissue specificity, which is sometimes very diverse for the different AAV types [4; 5].

An *inverted terminal repeat* (ITR) delimits the genome at the 5' end and at the 3' end, respectively. The ITRs contain the *cis-acting* elements for the replication of the viral genome and its integration into the host genome as well as the packaging signal. For productive, lytic infection, AAV require helper functions provided by helper viruses (such as adenovirus, herpes simplex virus type I and type II, cytomegalovirus or human herpesvirus-6). In the absence of helper functions, a cell is infected by AAV, but the transferred AAV genome rests in the nucleus of the host cell (latent infection), mainly as an extrachromosomal episome. However, it may also be present integrated in the host genome [6; 7]. In humans, AAV DNA is the only known viral genome to be specifically integrated into the host cell genome. This integration usually occurs in the AAVS1 locus of chromosome 19 [6]. The latent virus can be remobilized by overinfection with adeno- or herpesviruses.

AAVs are presumably transmitted via the respiratory tract and faecal-oral transmission [7; 8]. The more than one hundred AAVs isolated from primates to date can be divided into 13 serotypes [5; 9].

In this context, serotypes **AAV-2, -3, -5, -6** and **-9** were isolated from humans [5; 10; 12].

In contrast, serotype **AAV-1** has been isolated from both monkeys and humans [5].

Serotypes **AAV-4, -7, -8, -10, -11, -12** and **-13** have been isolated from monkeys [5; 11-14].

Depending on age and geographical region, about 80% of people have antibodies against AAV [2]. These show some cross-reactivity to other AAV serotypes [6]. Overall, seropositivity for AAV-1, -2 and -3 has been described for 70% of people studied [2; 15-17]. Approximately 45 % have antibodies to AAV-5 and -6 [2; 15]. Slightly lower (~ 38 %) is the seroprevalence for AAV-8 and -9 [2; 15-17]. The seroprevalence for AAV-7 is about 10 % and for AAV-4 less than 2 % [16; 18]. The seroprevalence for serotypes AAV-10, -11, -12 and -13 are unknown.

Despite the ubiquitous distribution of AAV and the high infestation, no AAV-associated diseases are known to date in humans or animals, which is why it is assumed that AAV are apathogenic

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[2; 6; 7]. In addition, a protective effect of AAV, for example against the development of tumours/cancerous diseases, is discussed [19; 20].

In 2001, AAV serotypes AAV-2, -3, and -5 were assigned to risk group 1 by the ZKBS, and AAV serotypes AAV-1, -4, and -6 to -12 were assigned to risk group 2. Furthermore, **AAV-3b, an** isolate derived from **AAV-3, was assigned to** risk group 1. **AAV-rh10** was isolated from rhesus monkey tissue in 2003 [4] and assigned to risk group 2. In a later study, 59% of humans were shown to have antibodies to AAVrh10 [21].

In TRBA 462 "Classification of viruses into risk groups", AAV serotypes 2, 3 and 5 are classified in risk group 1. AAV serotypes 1, 4 and 6 - 11 are classified in risk group 2.

The Dutch *Commissie Genetische Modificatie* (COGEM) recently classified the serotypes AAV-10 to -12 and AAVrh10 in risk group 1 [22]. In addition, it recommended that all AAV of the species *Adeno-associated dependoparvovirus A* and *B should* generally be assigned to risk group 1. This was justified by the ubiquitous distribution, the dependence on a helper virus and the absence of any evidence of pathogenicity.

The *US National Institutes of Health* (NIH) has classified all AAV serotypes as non-pathogenic risk group 1 [23].

Risk Assessment:

AAV are widely distributed in a wide variety of vertebrates, including humans, due to their high infectivity. However, due to the high seroprevalence for various AAV in humans, infection is not thought to be associated with pathogenicity. For some AAV serotypes, no natural infections in humans have been described, and seroprevalence is also low or unknown. Pathogenicity is also very unlikely for these AAV, but ultimately cannot be ruled out with certainty.

Based on the new findings since the ZKBS opinions on the classification of AAVs of 2001 and 2005, downgrading of individual AAV serotypes is carried out.

The ZKBS recommends the following classification of the various AAV serotypes as donor and recipient organisms for genetic engineering work in accordance with § 5 Paragraph 1 GenTSV in conjunction with the criteria in Appendix 1 GenTSV:

AAV-1 through -3, AAV-3b, AAV-5, AAV-6, AAV-8, AAV-9, and **AAV-rh10:** **Risk group 1**

AAV-4, AAV-7, AAV-10 to -13: **Risk group 2**

Notice:

If appropriate data are provided, downgrading of further AAV serotypes may be possible.

AAV-derived vector systems

AAV-derived vectors are infectious, replication-defective particles with DNA portions of AAV that can transmit foreign DNA.

Recombinant AAV vector particles are now being tested in numerous clinical trials as therapeutics for a wide variety of diseases [5; 19]. The first AAV-based drugs have already been approved in Europe (Glybera®, Luxturna™).

A conventional system for producing recombinant AAV vector particles consists of two plasmids, usually derived from pBR322, and a helper virus [6]. The transfer plasmid carries from AAV only the ITRs located upstream and downstream of the nucleic acid segment to be transferred. On the helper plasmid, only the nucleotide sequences of the reading frames *rep* and *cap* are present from AAV. There is no overlap of homologous AAV nucleotide sequences between transfer and helper plasmid, thus homologous recombination is not to be expected.

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To produce AAV vector particles, host cells are co-transfected with transfer and helper plasmid and infected with a helper virus, as the latter provides the essential viral helper functions to propagate AAV.

In more advanced systems for the production of AAV vector particles, the viral helper functions are provided independently of the helper virus on a third plasmid, so that infection with a replication-competent helper virus is no longer necessary [6]. This also prevents the production of replication-competent helper viruses. Of the adenoviruses previously used as helper viruses, the E1a, E1b, E2a, E4 and VA proteins are necessary for the production of AAV vector particles. By using the 293T cell line, which provides the adenoviral E1 proteins, only the genes for the adenoviral proteins E2a, E4 and VA are required on the helper plasmids, in addition to the *rep* and *cap nucleotide* sequences [24].

In numerous clinical studies with AAV vector particles of different serotypes in humans, it has been shown that the vector DNA does not enter the germ line [25]. Excretion of AAV vector particles by subjects, for example via urine and saliva, occurred depending on the administration route and dose [25]. However, excretion is low-level and transient, thus limiting the spread of infectious AAV vector particles [26].

Within the host cell, the transfer DNA is mainly extrachromosomal.

The vector particles are replication-defective and, apart from the AAV ITR, no other genes of AAV or the helper viruses are present on the transfer vector. In addition, the vector-receptor systems described under risk assessment points 2 and 4 comply with biological safety measures according to Section 8 (1) and (2) GenTSV.

Risk Assessment:

1. Recombinant AAV vector particles containing no AAV nucleic acid segments other than the ITRs and, in addition, only nucleic acid segments with no hazard potential shall be classified in **risk group 1,** even if they are pseudotyped. The classification is independent of the AAV from which the ITRs used originate. Genetic engineering operations involving genetically modified organisms that meet the above criteria are to be assigned to **safety level 1.**
2. Cells or cell lines of risk group 1 that have been transduced with the recombinant AAV vector particles mentioned in point 1 are classified in **risk group 1**. Genetic engineering operations involving genetically modified organisms that meet the above criteria shall be assigned to **safety level 1.** In the case of cells that deliver organisms of higher risk groups, the hazard potential of the organisms is fully taken into account in the risk assessment.
3. Recombinant AAV vector particles that do not contain AAV nucleic acid segments other than ITRs, but additionally contain nucleic acid segments with neoplastic transforming potential, shall be classified in **risk group 2**. The classification is independent of the AAV from which the ITRs used originate. Genetic engineering operations involving genetically modified organisms that meet the above criteria are to be assigned to **safety level 2.**

   Cells or cell lines of risk group 1 that have been transduced with the recombinant AAV vector particles mentioned in point 3 are classified in **risk group 1**. Genetic engineering operations involving genetically modified organisms that meet the above criteria shall be assigned to **safety level 1.** In the case of cells that deliver organisms of higher risk groups, the hazard potential of the organisms is fully taken into account in the risk assessment.

5. If AAV vectors are to be used to transfer prion or toxin genes, a case-by-case evaluation by the ZKBS is required.

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Notes:

1. In the case of contamination of the recombinant AAV vector particles described in risk assessment point 1 with helper viruses, the risk potential of these viruses is fully taken into account in the risk assessment.

2. If there is a possibility that overlapping AAV nucleic acid segments on the transfer and helper plasmid give rise to replication-competent, possibly chimeric AAV, or if such AAV are deliberately generated, the AAV from which the nucleic acid segments for the Rep proteins are derived shall be relevant for the risk assessment.
3. Experimental animals whose somatic cells have been transduced using recombinant replication-defective AAV vector particles are not GMOs. However, the animals must in principle be regarded as carriers of GMOs. The time during which infectious vector particles remain in the animal is highly dependent on the dose administered and the route of inoculation. In addition, the animals may shed the administered vector particles. However, if it can be demonstrated with the aid of data or suitable literature for comparable systems that no more AAV vector particles are released from the treated animal after a certain period of time, it is harmless from a safety point of view for the Land authorities to set appropriate time limits on their own responsibility after a case-by-case assessment, after which the animals are no longer treated as carriers of GMOs.
4. It can be assumed that the protection of the legal interests according to § 1 No. 1 Genetic Engineering Act is also ensured if the animal cadavers of test animals inoculated with AAV vector particles of **risk group 1,** for which corresponding data of Note 3. were not submitted, are disposed of without prior autoclaving as is customary in laboratory animal husbandry. Unless Section 24(1)(3) of the Genetic Engineering Ordinance already applies, the competent licensing and supervisory authority may decide, in accordance with Section 2(2) of the Genetic Engineering Ordinance, that these animal carcasses may be disposed of in the normal way in laboratory animal husbandry without autoclaving if it is ensured that the animal carcasses do not enter the food and feed chain.
5. In the case of laboratory animals that have been inoculated with AAV vector particles of **risk group 2** and for which the data mentioned under Note 3. have not been submitted, it can be assumed that a clear depletion of the AAV vector particles has been achieved seven days after inoculation, so that work with the cadavers of these animals does not present any hazard potential exceeding safety level 1. The protection of legal interests according to § 1 No. 1 of the Genetic Engineering Act is also ensured if the animal cadavers are disposed of in the usual way in laboratory animal husbandry without prior autoclaving. Unless Section 24(1)(3) of the Genetic Engineering Ordinance already applies, the competent licensing and supervisory authority may decide in accordance with Section 2(2) of the Genetic Engineering Ordinance that these animal cadavers may be disposed of without autoclaving as is customary in laboratory animal husbandry, if it is ensured that the animal cadavers do not enter the food and feed chain.
6. Parvoviruses are very insensitive to alcohol-based disinfectants. However, because of their better tolerance or lower toxicity, only hand disinfectants whose efficacy is based on alcohols are available. For this reason, hand disinfectants effective against AAV are not available [27]. Therefore, during genetic engineering work with AAV or AAV vectors of risk group 2, disposable protective gloves must be worn and changed regularly, and hands must be washed frequently. When selecting surface disinfectants, it should be taken into account that the effectiveness of the agents has also been tested against parvoviruses. This is the case for disinfectants that are listed in the list of disinfectants and disinfection methods tested and approved by the Robert Koch Institute and in the disinfectant list of the Association for Applied Hygiene with the efficacy range "virucidal".
7. If AAV vectors are to be used to transfer nucleic acid segments with neoplastic transforming potential, the following general statements of the ZKBS are referred to in this context:
    - Statement of the ZKBS: Precautionary measures when handling nucleic acids with neoplastic transforming potential (ref. 6790-10-01, updated December 2016)
    - Recommendation of the ZKBS on adenoviral and AAV-derived replication-defective particles that transfer a nucleic acid segment with neoplastic transforming potential (Ref. 6790-10-83, updated April 2020).

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