The Challenges of AAV-Based Gene Therapy

A lack of suitable and widespread reference material in gene therapy is just one of the many obstacles facing laboratories around the world attempting to find treatments for genetic diseases

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Adeno-associated virus (AAV) has become one of the most popular vectors for gene delivery in various *in vivo* gene therapy applications. Hundreds of projects are ongoing using the various serotypes with a significant fraction of those in clinical studies. In the case of a localised application, e.g., in the eye, a single small dose depending on the organ or tissue of e.g., ~1E11 vector genomes of AAV is sufficient. Whereas for a systemic treatment of certain genetic diseases like muscle dystrophies or haemophilia, high dosing at or above ~1E14 viral genomes per kilogram (vg/kg) is required.

The Challenge of Finding the Right Dose

Finding the right dose without causing severe side effects is a major challenge in every therapeutic approach. It is an even greater challenge in the area of viral-based gene therapy due to the limitation of a single vector application to each patient.

In a study led by James Wilson's group in the Gene Therapy Program at the University of Pennsylvania, US, it was demonstrated that a high AAV load in the range of 1E14 vg/kg or higher could cause severe hepatotoxicity in piglets and non-human primates (NHP) (1).

Some of the clinical trials initiated in the past couple of years had to be suspended due to severe adverse effects. Most of these side effects have been reversible (Clinical Trial ID NCT03368742), however, some were also associated with the death of some patients (NCT03199469) (2-3). One commonality of these studies was the use of a vector concentration in the high dose range of >1E14 vg/kg.

A particularly tragic example was reported during the clinical trial for X-linked myotubular myopathy (NCT03199469) using high doses of the AAV serotype 8 (3E14 vg/kg) that was associated with the death of three patients participating in the high-dose cohort of the trial (2-3). In contrast, the lower-dose cohort in this trial received 1E14 vg/kg and did not show any severe effects. Investigations are ongoing to clarify a potential link between the therapy attempt and the hepatotoxicity observed while the trial was put on hold. Interestingly, this particular clinical trial (NCT03199469) was also mentioned in the study of James Wilson (1). It was stated that no adverse effect could be observed using exactly this AAV8 vector at 8E14 vg/kg in a corresponding pre-clinical trial in NHPs. Therefore, not only high dosage but also pre-existing conditions might have contributed to the liver dysfunction shortly after receiving the AAV treatment (2). However, it is difficult to conclude from the data if there is a principal issue with the transferability of toxicity studies from animals, including NHPs, or if there is a problem with the comparability of titers in different studies and labs. If the latter is part of the problem, finding the right dose will remain a major challenge.

Comparability – A Major Issue

The dose used in clinical studies is usually indicated as copy number of vg/kg. The well-known high inter-lab variability of the quantitative polymerase chain reaction (qPCR) method used to determine the vg titers makes it substantially difficult to compare the doses of different vectors used in different studies. Vector copy numbers of the same vector could have >50% higher or lower vg titer values when measured in different labs as shown by the

variability for the American Type Culture Collection (ATCC) reference standard material (RSM) (see **Table 1**).

RSM (ATCC)	CV (qPCR)*
rAAV2	78%
rAAV8	113%

*Coefficient of variation (CV) for qPCR; data from (4-5)

Table 1: Variability of qPCR data for ATCC RSMs

It is further known that the production of different serotypes with different protocols can lead to a significant fraction of empty viral capsids in the final drug preparation. Therefore, the number of assembled viral capsids and the corresponding protein in mg/kg injected into the patient could be significantly higher than the measured vg/kg dose determined by PCR methods. With the doses of some of the trial therapies close to or even at the level associated with toxic effects, a careful and comprehensive determination of particle titers including the vg titer is, therefore, crucial.

Nevertheless, comparability between different labs and serotypes is difficult because of the variability of the quantification assays available and the lack of RSM for most of the relevant serotypes. So far, only AAV2 and AAV8 RSMs are available from the ATCC (4-5).

As it is difficult to compare viral genome titers from different serotypes used in the same therapeutic approach or the same serotypes used for different therapies, it is a major challenge for regulatory authorities to get a thorough understanding of thresholds needed to evaluate the safety of gene therapies.

Orthogonal Methods for Characterisation

Multiple orthogonal methods should be applied for a comprehensive characterisation of AAV-based gene therapeutics. The combination of several methods could allow reliable quantitative measurements of virus particles based on established protocols in the area of small molecules/protein and peptide chemistry.

The calibration of AAV enzyme-linked immunosorbent assay (ELISA) was performed using internal reference material characterised by electron microscopy (negative staining and cryogenic electron microscopy [cryo EM], discriminating full and empty particles) and qPCR similar to the AAV2 and AAV8 RSM protocol. No significant difference between negative staining and cryo EM was observed in all of the internal standards for AAV1, AAV3, AAV5, AAV6, AAV9, and AAVrh10 (see **Figure 1**). The qPCR was performed in three different labs (two qPCR and one droplet digital PCR [ddPCR]) and average vg titer values were corrected by the factor of total/full capsid ratio to determine total particle titer.

Following the example of the AAV2 and AAV8 RSM characterisation, some of the ELISAs for other serotypes have been re-calibrated and corrected using the more reliable workflow described above compared to the earlier methods that were used to quantify AAV and accepted in the field in the past.

The Requirement for RSM

One of the obstacles for a thorough analytical characterisation of AAV-based therapeutics lies in the limited availability of material for repeated testing with multiple techniques. For comparison, a large-scale preparation of AAV from 100L of cell culture volume could yield around 1E16 viral particles corresponding to approximately 70mg of virus. This is roughly 7,000-fold lower than the yield of a typical therapeutic antibody production with around 5g of purified antibody per litre of culture. Based on the limited availability of AAV material, methods using small amounts of the valuable drug substance are preferred. However, any technique capable of measuring very low volumes of the analyte almost inevitably has issues with, for example, sensitivity to contaminants, or high dilution factors of the starting material causing high variability in the workflow, therefore leading to accuracy and reproducibility issues. Even if the issue of limited availability of material could be resolved by increasing production capacity and yield, the

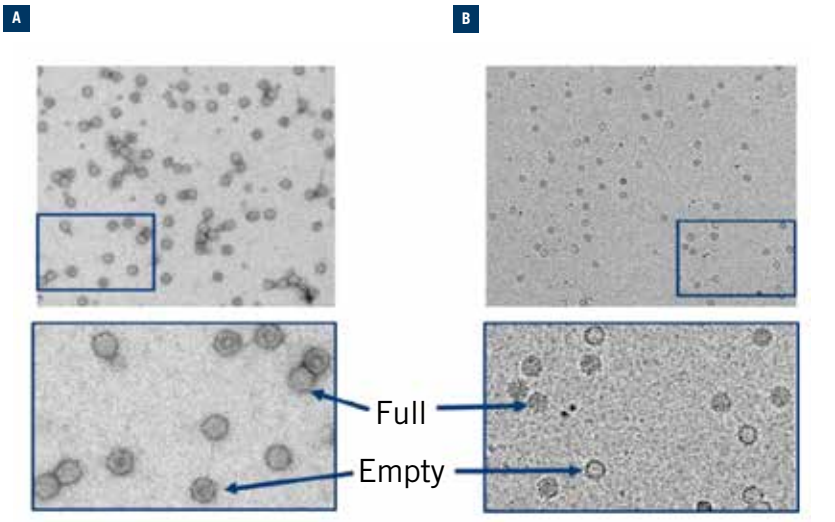

Figure 1: Negative staining electron microscopy (A) and cryo-electron microscopy (B) of AAV3

comparability of results will remain a challenge unless reliable orthogonal measurements and/or carefully defined reference standards are available.

Certainly, every lab working with AAV has generated their own carefully characterised internal reference materials using different methods and protocols with more or less variable results. Comparability of different virus products from different labs, however, remains difficult due to the production of individual reference materials. Therefore, reproducibility and variability of different methods need to be carefully evaluated to meet the substantial need for methods with low inter-assay and inter-lab variability. In the past few years, a shift from the classical qPCR (comparably high variability) to ddPCR with comparably less variability and no standard curve needed for the measurement of vg titers could be observed. Like all methods, with ddPCR, one needs to consider pitfalls that could cause systematic errors if new methods are not validated and tested thoroughly for correctness, but the focus is mainly on low variability between measurements. Ideally, a test of inter-lab variations of identical samples would enable the benchmarking of the lab-specific protocols as well as build trust in comparability of data from different sources. This is especially true for AAV serotypes where there is no independent reference material available.

With increasing usage of AAVs for gene therapy, even for the AAV2 and AAV8 RSMs, stability and – with the finite number of vials that were produced only once – availability in the future could become an issue. New batches for these two serotypes would need to be produced, characterised, and made available to the community in order to ensure the reliable quantification in the long run. An independent standard reference material for all AAV serotypes with a defined genomic and particle titer determined with the best currently available methods, accessible to the AAV gene therapy community is, therefore, required. This could follow the example of ATCC RSM for AAV2 and AAV8 in order to achieve better comparability and, ultimately, more safety due to more reliable dose determination.

This establishment of RSMs should be done in a concerted effort by as many stakeholders in the field as possible. The availability of RSM for the AAV gene therapy community will also simplify the assessment of safety by regulatory authorities to prevent severe adverse events for patients caused by future AAV vector-based drug substances.

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