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Title: **Standards for the Measurement of Pre-Existing Immunity to Adeno-Associated Virus Vector**

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Abstract

Executive Summary

Importance of gene therapy for patients

Many genetic therapies utilize adeno-associated virus (AAV) vectors as a gene delivery vehicle. Wild-type AAV is a small non-pathogenic virus known to infect humans as well as other primate species. As AAV causes a very mild immune response in humans, compared to other viral vectors, it is an attractive vectors for gene therapy. Although there are hundreds of genetic therapies in trials, only few are currently approved. For the patients and families of those diagnosed with debilitating or life threatening diseases, these genetic therapies hold a promise to change their quality of life.

To name one of those diseases, Duchenne is a rare X-linked genetic disorder, one of more than 7000 rare diseases identified nowadays. Duchenne primarily affects 1:4600 young boys who are often wheelchair bound by their teens and do not survive into their 30s. Currently genetic therapy trials are conducted to treat Duchenne that have the possibility to restore dystrophin, the missing or truncated protein, giving these children the possibility of preserving function and extending life span. As gene therapy trials open, families seek out these opportunities, willing to do whatever it takes to participate in these studies. However, due to exposure to wild-type AAV, potentially eligible participants are excluded from gene therapy trials due to the presence of pre-existing antibodies that neutralize the therapeutic vector.

Additionally, for subjects who meet enrollment criteria, these trials are sometimes not repeatable. For example, if the therapy is not successful, re-dosing a patient within the same AAV vector or enrolment in another AAV-based gene delivery trial is not always possible due to the development of immunity to AAV following the vector infusion.

Although there is no removing all the risks and burdens the participants in new therapy trials face, stakeholders have the responsibility to ensure that we maximize the learnings deriving from well designed trials in which all parameters are collected in a standardized and comparable manner. To this aim, a clear and reliable testing method for preexisting immunity to AAV is needed. Currently companies develop viral based approaches using different methodologies, with different cutoffs for gene therapy trial exclusion criteria when it comes to pre-existing humoral immunity to the vector. While a clear scientific rationale is clearly behind these criteria, it is also important to confidently and clearly explain the rationale or the variability in parameters to patients and their families. Together key stakeholders from industry and academia can work together toward standardized methodologies and guidelines to measure anti-AAV immunity and to educate the medical and patient community on the meaning of being positive or negative for anti AAV antibodies.

Importance and Benefits of Standardized Measurements of Pre-existing Immunity and the Impact on Gene Therapy

Introduction into AAV

The development of Adeno Associated Viruses (AAV) as vectors for the delivery of gene therapies has seen significant clinical success recently for the treatment of congenital monogenic disorders and has rapidly become the leading platform for gene delivery for the treatment of a variety of human diseases (Naldini L. Nature 2015; 526: 351-60, High and Roncarolo, NEJM 2019). AAV were first

discovered in 1965 by Atchison and colleagues as a contaminant in preparation of Adenovirus and were determined to be antigenically distinct defective viral particles unable to replicate independently in human cells (Atchison et al. Science 1965;149:754-756 and reviewed by Hastie and Samulski. Human Gene Therapy 2015;26:257-265).

AAV are now known to be among the smallest and simplest virus yet discovered, classified as a Dependovirus in the family Parvoviridae, they are single-strand DNA viruses comprised of a ~5kb genome. The AAV genome contains two large open reading frames, rep and cap, bookended by self-priming, palendromic inverted terminal repeats (ITR). AAV genomes of both polarities are packed into a ~25nm icosahedral vector particles comprised of three coat proteins, VP1-VP3, in a 1:1:20 ratio respectively (reviewed by Gao. Current Gene Therapy, 2005, 5, 285-297 and by Hastie and Samulski. Human Gene Therapy 2015;26:257-265). As vectors for use in gene therapy, the AAV genome is modified by removal of the rep and cap genes which are provided in trans for packaging, and replaced by the transgene cassette encoding the therapeutic protein of interest flanked by the two ITRs.

Several early primate and human isolates of AAV have been extensively utilized as gene therapy vectors including AAV-1, -2, -5, -8 and -9, and more novel serotypes continue to be discovered in primate and non-primate species including equine, ovine, avian, bovine, caprine and snake (Reviewed in Arbetman et al. J.Virol. 2005;79(24):15238-15245). Throughout life people are naturally infected with AAVs which evoke an immune response and each individual therefore has a unique pre-existing humoral immunity to AAV vectors. This pre-existing immunity may recognize a related therapeutic AAV and neutralize its ability to transduce host cells and limit their clinical utility. There are several AAV serotypes, including AAV-1, -2, -5, -6, -8, and -9 and their derivatives, that have to date been commonly used in existing gene therapy programs and are frequently referred to as “common AAVs". The seroprevalence of immunoreactivity for the more common AAVs that can impact on the success of the gene therapy outcome can vary significantly by AAV serotype and geographic location, but generally ranges from a low of 20% for AAV5 and up to 60% for AAV2 (Boutin S. 2010 Hum Gene Ther 21: 704-712. Calcedo R, 2009. J Infect Dis 199: 381-390. Louis Jeune V. 2013. Hum Gene Ther Methods 24: 59-67).

Finally, there is often antibody cross-reactivity among the various serotypes where previous exposure, either to natural infection or previous gene therapy dose administration, stimulates an antibody response that can neutralize multiple serotypes. Early classification of AAVs was traditionally done through serology. A unique serotype was defined as a virus that cannot be efficiently neutralized by serum generated against viruses with known serotypes (Gao 2005, Current Gene Therapy). However more recent next-generation sequencing-based analysis of viral sequences provides a more precise phylogeny.

Cross-reactivity is typically measured in a neutralizing antibody assay and has been most systematically studied by Gao et al. 2004, where rabbit polyclonal antibodies against AAV serotypes 1 to 9 were generated and evaluated for cross neutralization of other serotypes. This work largely confirmed the serological distinctiveness of the common AAV serotypes with low titer cross-neutralization capacity. Importantly, data on cross-reactivity and cross-neutralization in clinical subjects remains sparse, and assessment of vectors for gene therapy applications and selection of appropriate serotypes will require independent evaluation in specific disease populations.

Scope of this paper - (likely will end up in the exec. summary)

Neutralizing Antibodies (NAbs) and Vector Transduction Efficiency

Adeno-associated virus (AAV) neutralizing antibodies (NAbs) are anti-capsid antibodies that target epitopes critical for viral infectivity, and by extension, critical for transduction by AAV vectors. Some of these antibodies have been shown to target overlapping regions in various AAV serotypes and occlude receptor binding sites, like the heparan sulfate proteoglycan (HSPG) site on the AAV2 and AAV6 capsids (Opie SR et al., 2003; Wu Z et al., 2006) or a sialic acid binding site in AAV5 (Excoffon

et al., 2009). Since these antibodies compete for receptor attachment sites present on target cells, they can hinder interactions with the vector.

NAbs against AAV vector capsids can decrease transduction efficiency, especially when vectors are systemically delivered. Therefore, detection of pre-existing NAbs to AAV often constitutes an exclusion criterion in liver-directed gene therapy trials. Multiple previous studies of AAV-mediated gene therapies, both clinical and non-clinical, have shown that pre-existing neutralizing antibodies (NAbs) can interfere with AAV vector transduction *in vivo* and thus limit therapeutic efficacy. The impact of NAbs on vector transduction was already observed in the first clinical trial of liver-directed gene transfer. In this trial, an AAV2 vector encoding for coagulation factor IX (F.IX) was administered to severe hemophilia B patients (Manno et al., 2006). Despite receiving the same vector dose, patient E, who had an anti-AAV2 NAb titer of 2, expressed peak levels of F.IX transgene of only ~11% of normal while patient F, who had a NAb titer of 17, did not have any detectable circulating F.IX. Similarly, animal studies showed that even low NAb titers can prevent liver transduction in mice (Scallan et al., 2006) and non-human primates (NHP) (Jiang et al., 2006; Long et al. 2019).

Of note, the presence of anti-AAV NAbs is not exclusively an issue associated with the intravenous route of vector infusion. Anti-AAV antibodies also affect transduction efficiency when the vector is administered directly to extravascular body compartments such as the joint space (Boissier et al., 2007; Mingozzi et al.,2013) or the cerebrospinal fluid where antibodies can also be present (Gray et al., 2011; Haurigot et al., 2013) though at lower levels compared to the systemic circulation. Conversely, anti-AAV antibodies do not appear to significantly block transduction if the vector is directly injected intramuscularly (Brantly et al., 2009; Manno et al., 2003; Stroes et al., 2008) or into the brain (Kaplitt et al., 2007). For other “immune privileged” sites of the body, such as the eye, AAV injection into the subretinal space in animal models (Amado et al., 2010) and humans (Bainbridge et al., 2008; Bennett et al., 2012; Cideciyan et al., 2008; Maguire et al., 2008) was also not affected by pre-existing NAbs. However, transgene expression after intravitreal administration of AAV was inhibited in the presence of NAbs (Kotterman et al., 2015; Desrosiers et al., 2018).

Consistent measurement of anti-AAV NAb titers remains challenging and the field strives for continuous improvement of existing methods. While multiple approaches are being developed to overcome the limitation of pre-existing antibodies to AAV and allow for enrollment in trials of seropositive individuals because the exclusion of patients with pre-existing anti-AAV antibodies remains a frequent eligibility criterion, due to the anticipated decreased treatment efficacy. Patient impact from pre-existing AAV immunity is amplified, as these patients often have no other treatment options. Understanding of the impact of pre-existing anti-AAV antibodies and access to reliable fit-for-purpose methods to detect whether a patient is positive for pre-existing anti-AAV antibodies is therefore critical.

Total binding vs. Neutralizing AAV antibodies

Current methodologies applied to detect and evaluate humoral immune responses to AAV vector-based gene therapies (GTx) can be broadly divided into two categories: Total binding antibody (TAb) and Neutralizing antibody (NAb) detecting methods. The TAb methods assess the presence of any immunoglobulins, of any isotype, that specifically bind to any of the epitopes present on the surface of the AAV capsid. The binding of non-neutralizing TAb to the capsid can lead to enhanced vector uptake by immune cells and elimination (Ref missing). Depending on antibody binding affinity and/or the nature of targeted AAV capsid epitope, the antibody may also effectively inhibit the interaction of the vector with cellular receptors or block other steps involved in internalization and processing, resulting in overall neutralization of the vector transduction. A subset of TAb antibodies with these neutralizing qualities are thus referred to as NAbs.

The fundamental difference between assays used to detect TAbs and NAbs is that the former can assess only the event of binding of AAV capsid-specific antibodies and are not designed to determine the potential impact on the complex and multi-step vector transduction process. Conversely, typical

cell based in vitro NAb assays are designed to detect vector transduction inhibition which may be mediated by factors other than immunoglobulins [refLFalese et al. 2017, Long et al. 2019]. Generally, there is a good correlation between the presence of binding (TAb) and neutralizing (NAb) antibodies, although not in samples containing lower antibody titers (Falese L. 2017; Veron P et al., 2012). Recent reports also found some evidence that some AAV binding antibodies may actually lead to a small increase in liver transduction, mediated by an unknown mechanism (Fitzpatrick et al., 2018).

NAb assays are designed to determine the presence of factors that are capable of inhibiting cellular uptake of the vector and/or the expression of the transgene. It is important to point out that most NAb assays broadly detect any transduction-inhibiting factors, since these assays are not specific for antibodies, unless a confirmatory antibody-identification step is implemented (further discussed below). Of note, TAb and NAb methods used to determine the presence of pre-existing AAV antibodies may also be applied to evaluate the immune response *after* administration of the gene therapy.

Assay Types

Assay methodologies and analytical platforms that are commonly applied for detection of anti-biotherapeutic drug antibodies (ADA) can be seamlessly transformed to produce assays aiming to detect anti-AAV TAbs. As such, **ligand binding assays (LBA**) have become one main approach to detect TAbs. In these assays, the TAb analyte is complexed with specific AAV vector capsid and then detected by an assay reagent that is conjugated to an analytical platform specific label, followed by a readout step. With a variety of LBA platforms available, two are typically applied: Enzyme Linked Immunosorbent Assay (ELISA) and Electrochemiluminescence (ECL). The latter is often executed on a Meso Scale Discovery (MSD) instrument.

Common TAb assay setups include bridging and direct binding formats **<BG - i can provide a cartoon/figure of these specific types of LBA>**. In the bridging assay format, TAb detection is based on the multi-valency of IgM and most IgG isotypes. While one of the arms of the TAb binds to a solid phase (e.g. plate) immobilized AAV vector, the other arm of the immunoglobulin can bind to a labeled AAV vector reagent. In a direct binding assay format, human or other species origin TAb bound to a solid phase immobilized virus is detected by an anti-species immunoglobulin specific reagent, e.g. anti-human IgG/M antibody, or by another immunoglobulin detection reagent such as labelled Protein AG/L (Long et al.). Other LBA formats can be applied for detection of TAbs, similar to other formats that have been applied for detection of ADA [ref – e.g. for SPR for ADAs].

In summary, TAb assays are much easier and quicker to perform than NAb assays. Although still technically challenging, they require less specialized skills. A harmonization in the measurement of anti-AAV antibodies in TAb assays may enable better comparisons of pre-existing titer levels between studies. This would enhance our understanding of the impact of anti-AAV antibodies on gene therapy outcomes.

The most broadly used method to detect anti-AAV NAbs are **in vitro cell-based assays** employing a vector capsid for a particular AAV serotype that carries a reporter gene (Boutin et al., 2010; Calcedo et al., 2009; Mingozzi et al., 2013; Moskalenko et al., 2000). Cells are transduced with the reporter vector in the presence of serum or plasma, and thus the presence of NAbs is inversely correlated with the magnitude of reporter gene expression. A number of reporter gene proteins have been proposed, including green fluorescence protein, LacZ and luciferase. The luciferase reporter gene is often viewed as preferred choice and can likely allow for a more sensitive read out [ref].

Although, cell-based NAb assays are only semi-quantitative and neutralization titers are defined as the sample dilution that results in 50% or greater reduction of cellular transduction by the AAV reporter vector, they are currently the most valuable tool to predict the efficacy of AAV transduction *in vivo* for majority of the AAV serotypes used in clinic. A statistically based approach to define assay cut-point has been also discussed [ref Falese et al., 2017] and would be more in line with regulatory guidance on quantitative immunogenicity assays (FDA 2019). Cell based assays are known for their

relatively high variability. Assay variability may be particularly important to appreciate when testing samples containing very low amounts of NAb.

It is essential to note that the use of a reporter gene carrying AAV vector is clearly different from the final therapeutic AAV vector that contains the treatment-specific transgene. In addition, the reporter AAV vector may also have been purified and formulated differently than the therapeutic vector. The potential impact of such differences on the interpretation of NAb assay results needs to be considered.

Further, a 2017 study of non-human primate showed that not all AAV transduction inhibiting factors detected by a NAb assay led to decreased efficacy *in vivo* (Long B. et al.). Specifically, transduction inhibition titers measured in a cell based NAb assay in the absence of detectable AAV antibodies in an orthogonally performed TAb assay showed no evidence for inhibited transduction *in vivo*. This observation can be interpreted in three ways. First, the existence of non-antibody factors that efficiently block the transduction of a target cell line *in vitro* but are inefficient *in vivo*. Second, it could be an issue of vector dose, hemodynamic, presence of empty capsids in the therapeutic vector preparation due to differences in manufacturing processes. Third, the observed inhibition of cell transduction *in vitro* might have been caused by nonspecific matrix effects. Matrix effects are common when testing serum or plasma samples in multiple bioanalytical assays and across different species. In cell-based assays, it may be caused by compounds contained in a sample that do not interact with the AAV capsid surface but can interfere with the assay readout in other ways. For instance, samples may contain a compound that is toxic to the target cell line or influences the promoter activity of the reporter gene, which could decrease transgene expression and thus give a false positive result.

A potential solution to overcome matrix interference and confirm that an assay has the ability to detect neutralizing antibodies in cell based AAV NAb assays is to implement an additional specificity step. In additional test wells, concurrent incubation of test sample can be performed first, with empty AAV capsid, devoid of reporter gene, and then with the reporter vector. If the decreased transgene activity is caused by the capsid-specific antibodies, they would be adsorbed on the empty capsid and the reporter vector, added in the second step, and would be able to effectively transduce the cells. On another hand, if the decreased transgene activity is caused by a factor that does not bind the empty capsid, but for example decreases viability of cells, the transgene activity will remain low in these wells (Kuranda et al. 2019 EAHAD abstract). Therefore, this additional test facilitates a distinction between AAV-binding transduction inhibitors and other compounds that do not bind the AAV capsid but interfere with assay readout. An alternative solution to increase the specificity of AAV NAb assays could be the implementation of a confirmatory antibody-depletion step, such as depletion of AAV antibodies using Protein AG/L sepharose, followed by a retest of the sample in the cell-based NAb assay. Samples with neutralizing AAV antibodies are expected to show increased AAV transduction after Protein AG/L depletion. This approach was previously used to unambiguously identify neutralizing ADA antibodies in a cell based NAb assay (Sanchez Gupta et al.).

Even though AAV TAb assays typically have similar or even higher sensitivity than cell-based assays, it is possible that certain epitopes on the AAV vector capsid are masked by the molecular label attached to the capsid-based detection reagents used in bridging TAb assays. This may preclude some antibodies from being detectable in TAb assays, while they could still mediate transduction inhibition in a cell based NAb assay where unlabelled AAV reporter vectors are used.

Recently it has been confirmed that neutralizing antibodies decrease binding of AAV vectors to the cells, and this property of NAbs was used to develop an assay where the quantity of AAV bound to the cell surface, which is inversely correlated with the amount of NAbs in samples, is measured by quantification of AAV genome copy numbers (Guo et al., 2019). While this assay is quick and easy to perform, it may underestimate the presence of antibodies able to inhibit vector transduction via other mechanisms. Indeed, some neutralizing antibodies do not block AAV entry to the cells but rather interfere with later steps such as intracellular processing that occurs after cell attachment but prior to

nuclear entry (Gurda 2012; McCraw et al., 2012). Thus, AAV transduction inhibition may involve post-entry events during endosomal trafficking and stabilization of the capsid against structural changes required for uncoating.

In addition to cell based methodologies, in vivo systems have been used for passive immunization-type studies: patient plasma is transferred into immunodeficient strains of mice which are then treated with the AAV vector to measure the neutralizing capacity of the transferred human plasma against the AAV reporter construct [ref]. This assay type offers no sensitivity advantage over TAb and cell-based NAb assays and is generally very cost-intensive, highly variable, and difficult to implement for large-scale testing.

Data reporting

The results from both TAb and NAb assays are reported as categorical results of either 'positive' or 'negative', based on whether the sample generated a response above or below the assay-specific cutpoint. In addition, for positive samples, a numerical titer value may be reported to indicate the magnitude of AAV binding or AAV neutralization capacity.

The binding titers reported by TAb assays are typically higher than those reported for NAb assays [ref]; however, a direct comparison of titer values is confounded by the different sensitivity of the methods used to detect both types of antibodies. NAb detecting assays are methodologically more variable, and results are variously reported as levels of neutralizing antibodies, neutralizing titer, neutralizing factors or transduction inhibition (TI) titer. Given that the reported values reflect different assay methodologies, assay sensitivities, and modes of data analysis, the utility of comparing NAb titer results across studies is very limited.

Addressing limitations and uncertainties

Given their less complex format, TAb assays may be more feasible to harmonize across laboratories than NAb assays, at least for a given AAV serotype. However, many critical reagents that affect assay performance and sensitivity may still introduce considerable variability in results. To achieve standardization of assays to measure pre-existing AAV immunity, one could try and seek industry-wide agreement to use a defined set of reagents, such as a particular cell line (e.g. HEK 293), helper virus, full or empty AAV capsids, sample dilutions, and negative control matrices, at least for detecting pre-existing AAV immunity for a particular serotype. In an ideal world, all gene therapies for a particular serotype may even be able to use the same screening assay provided by a third party vendor. However, given the vast diversity of how TAb and cell-based NAb assays are conducted across various companies and contract research laboratories, it may be very challenging to broadly harmonize assay methodology or reagent use. Therefore, it may be more helpful to establish anti-AAV antibody reference panels and develop regulatory guidelines as to how these methods should be validated, in order to standardize the basic performance characteristics required for these assays.

The Value of Standards to Address These Limitations

The sum total of all these technical issues will make adoption of a standard assay format difficult to achieve across AAV serotypes, and various groups have taken an approach to using methods tailored to their application. Cell based neutralizing assays are most in need of harmonization, but issues of cell types, preservation, standards and particular capsid infectivity compound the complexity. Certainly, from a standards perspective, there is a preference for LBA based methods to detect binding antibody, however it remains to be determined correlates between binding and neutralization assays. This dilemma arises from the fact that not all antibodies are neutralizing, and further, not all positive cell based NAb results correspond to antibodies that are detectable by LBA. The adopting of a standard-based approach may help to deconvolute these issues across AAV serotypes and disease indication.

Review of Current Standards

Although there are no physical antibody standards directly relating to the measurement of the pre-existing immunity to AAV, there are standards related to the use of AAV in regenerative medicine and standards for immunogenicity assessments of therapeutic proteins (Table 1). These standards are useful in defining the scope and value for the different approaches taken but they also highlight the need to address the lack of standards that specifically apply to pre-existing AAV immunity. For example, AAV capsid reference materials may be suitable standards for the quantification of AAV-based drug products.

However, using an AAV reference material when measuring pre-existing AAV immunity is unlikely to provide a practical solution to harmonize anti-AAV capsid antibody results obtained across different methods. The main reason for this is that there could still be different limits of detection or different titer calculation algorithms which are used in a method, which would impact the final reportable test result and numerical titer value. Further, cell-based NAb assays typically utilize specific AAV reporter genes that may not be present in AAV reference materials, thus precluding applying a true reference material in the assay. Moreover, many TAb methods to measure pre-existing immunity rely on modified AAV capsids, for example by attaching a molecular label either for the detection or capture steps. Any change made to the AAV reference material as required by a particular assay methodology would therefore render it unsuitable to serve as a universal standard. Finally, there is also an uncertainty as to whether the existing regulatory guidelines to evaluate the immunogenicity of protein therapeutics (FDA 2019) are fully applicable to evaluating pre-existing immunity against AAV capsids. The context of use for pre-existing AAV immunity measurements is for patient enrichment and enrolment stratification, based on the ultimate goal to predict treatment success. Therefore, guidelines for biomarker qualification and *in vitro* diagnostics may also need to be considered.

ID Number	Developing Organization	Title	Status
EMA/CHMP/ VWP/164653/2005	European Medicines Agency/Committee for Medicinal Products for Human Use	Guideline on Clinical Evaluation of New Vaccines	Published 2005
EMA/CHMP/ ICH/4409035/2009	European Medicines Agency/Committee for Medicinal Products for Human Use/ICH	ICH Considerations: General Principles to Address Virus and Vector Shedding	Published 2009
EMA/CAT/ 90193/2014,2018	European Medicines Agency/Committee for Advanced Therapies	Guidelines on the quality, non-clinical and clinical aspects of gene therapy medicinal products	Published 2018
EMEA/CHMP/BMWP/14327/2006 Rev 1	European Medicines Agency/Committee for Advanced Therapies/ Biological Monitoring Working Party	Guideline on Immunogenicity assessment of therapeutic proteins	Published May 2017
EP 2.6.35	EDQM	Quantification and characterization of residual host	Published

		cell DNA	2008
EP 5.2.12*	European Directorate for the Quality of Medicines and Healthcare (EDQM)	Raw materials of biological origin for the production of cell-based and gene therapy medicinal products	Published 2017
EP 5.14*	EDQM	Gene transfer medicinal products for human use	Published 2008
N/A*	FACT	FACT Standards for Immune Effector Cells (First Edition, Version 1.1)	Published 2018
FDA-2014-D-0663	FDA/CBER	Guidance for Industry: Determining the Need and Content of Environmental Assessments for Gene Therapies, Vectored Vaccines, and Related Recombinant Viral or Microbial Products	Issued 2015
FDA-2015-D-399	FDA/CBER	Guidance for Industry: Recommendations for Microbial Vectors Used for Gene Therapy	Issued 2016
FDA-2013-D-0092	FDA/CBER/CDER	Guidance for Industry Immunogenicity Assessment for Therapeutic Protein Products	Issued 2014
FDA Presentation	Division of Therapeutic Proteins OBP/CDER/FDA	The immunogenicity of therapeutic proteins- what you don't know can hurt YOU and the patient	Presented 2014
TR 47-2010	Parenteral Drug Association (PDA)	Preparation of Virus Spikes Used for Virus Clearance Studies	Published 2005
USP <1046>*	United States Pharmacopeia (USP)	Cell and Gene Therapies Products	Published; Currently Official USP41-NF36; 2013
USP <1047>*	USP	Gene Therapy Products	Published; Currently Official USP41-NF36; 2013
	FDA	FDA Guidance	
AAV Reference Materials			
ATCC	VR-1516		

	Adenovirus Type 5 Reference Material
	VR-1616 Recombinant Adeno-Associated Virus 2 Reference Standard Material (AAV2 RSM)
	VR-1816 Adeno-Associated Virus 8 Reference Standard Material (AAV8 RSM)

The Standards Coordinating Body

The Standards Coordinating Body for Gene, Cell, and Regenerative Medicines and Cell-Based Drug Discovery (SCB) is a non-profit organization that works to help advance the development of regenerative medicine therapies through the support, coordination, education, and establishment of standards. The SCB coordinates, prioritizes, and supports standards that advance process, measurements, and analytical techniques to support the global availability of products across a number of therapeutic sectors including gene therapy, cell therapy, cell-based drug discovery, and tissue engineering. SCB's mission is to coordinate the accelerated advancement and improved awareness of standard and best practices that address the rapidly evolving needs of the global regenerative medicine and advanced therapy community. To accomplish this mission, SCB operates through public-private partnerships with government agencies, regulatory bodies, and other government organizations involved in establishing consensus standards for regenerative medicine and other advanced therapy products. The SCB brings together product developers, tools and service providers, professional societies, government entities, and academic centers for the purpose of supporting standards development through coordination, prioritization, resource compilation, inter-laboratory data generation, joint participating in standards developing organizations (SDOs), education, and implementation of standards. With members from industry, professional societies, government and academic entities, SCB occupies a unique niche within the regenerative medicine ecosystem and has no vested interest in a particular scientific, commercial, clinical, or policy approach. SCB is focused on facilitating the use and development of standards in response to demonstrated need expressed by a range of stakeholders.

The SCB has conducted workshops and outreach events to further discuss, evaluate, and define the needs of the gene therapy field. Understanding these needs are critical in order to ensure that the standards that are established will both meet the gaps that exist today for users and regulators, as well as meet the requirements of the largest possible segment of the industry. A standard for the evaluation of pre-existing immunity to AAV vectors has repeatedly been identified by SCB stakeholders as needing development prioritization. This white paper is a first step in the process to reaching the goal of developing standards addressing this need.

Concluding Remarks

Recent demonstrations of the therapeutic benefit of AAV based gene therapy for the treatment of genetic disease has heightened the interest and hopes for this technology to provide viable, durable, safe and cost-effective treatment options for patients with a number of unmet, and poorly met medical needs [1,2]. In addition to the potential to develop novel therapeutic options for patients with significant medical need, the potential for patients to be free from the burden of routine drug administrations, concerns around sustainability of drug supply and sustainability of reimbursement associated with the costs of approved drugs is providing hope and opportunity to anxious and vulnerable patients and families in our communities. In order to meet the expectations of both patients and all associated

stakeholders, the field needs to continue to develop and evolve the tools needed to continue to ensure the highest level of patient safety and therefore facilitate appropriate design of clinical trials to improve the possibility of accelerated execution and increased technical success. To meet these expectations, it is critical for sponsors to ensure that the pre-established clinical trial enrollment criteria are adequately established and evaluated with the appropriate level of robustness and that those evaluations are homogenous between different trials and between different commercialized drug products. One area of current concern is the methodology and performance for quantitation of pre-existing neutralizing AAV antibody in potential patients, and how that factor may lead to the enrollment or rejection of patients due to inaccurate determination of nAb status

Who are the stakeholders and why should they care about standards development?

In addition to those patients and their families seeking therapeutic options, there are several stakeholders with a vested interest in ensuring the methodologies used to evaluate candidates for enrollment in clinical trials. Those stakeholders include academic investigators, drug developers, companion diagnostics (CDx) companies, patient advocacy groups, treaters, payers, regulatory agencies, investors, standards coordinating and development organizations and contract research organizations. While the shareholders have potentially different perspectives and biases, ultimately they will all benefit from improvements in the tools used to select the right patients to participate in clinical trials, including the methodologies used to screen for and quantify the presence of nAbs in candidates. Improving the selection of patients will ultimately ensure that only those patients with the potential to benefit from therapy would be exposed, but in addition the number of patients needed to demonstrate safety and efficacy would be reduced, the costs and duration of clinical trials would be reduced and the potential to accelerate the development and approval of the therapies would be increased. Investors would see faster returns, and payers would be confident that their clients needs are being adequately supported to justify the large costs of the therapies

How will standards improve nAb surveillance in clinical trials?

Establishing a strong suite of tools to quantify nAbs with a high degree of consistency between sponsors would reduce the heterologous nature of the results reported from ongoing trials. The technical basis of the analytical methods would be better defined, the significance of the findings of impact on patient safety and potential benefit from pre-existing nAb levels would be better understood and sponsors would have a higher degree of confidence in who they select for participation in their trials. In addition, having a better understanding of the impact of nAb would help define conditioning and mitigation treatments to potentially circumvent the impact of nAb and lead to provision of therapeutic strategies that are more universally applicable to patients with pre-existing nAb levels deemed refractory to current treatments.

Potential Next Steps

The standards coordinating body is committed to bringing the field together to develop appropriate strategy and actions to development suitable standards to improve the evaluation of pre-existing immunity in patients entering clinical trials. The development of suitable standards will require significant participation by all the stakeholders involved in this process. It will require collaboration on many levels, including technical, operational, sponsorship and data sharing to the extent that stakeholder interests are not violated. We urge active participation from all those with capabilities to enable the development these standards in a pre-competitive environment. The end goal is to provide the field with adequate tools to better address the deficiencies in current capabilities. For further information and to learn how your organization can participate, please contact the SCB at XXX.

One of the approaches in harmonizing methods relating to pre-existing anti-AAV immunity is to develop common principles for assessment in a position paper with joint input from regulators and industry, or as part of a formal regulatory guidance on assessment of immunogenicity for cell and

gene therapy products. The latter can be a lengthy and time-consuming process although it is likely to be the most favored option. This can help to standardize complex issues relating to selection of assay formats, required limits of detection, type of antibodies detected, cutoffs for positive samples, data analysis, etc …

The best possible approach for methodological standardization would be to agree on one particular AAV method principle and assay format. Even though this approach has been achieved for measuring neutralizing antibodies against IFN-beta [Wadhwa et al 2013], this would be rather difficult to achieve for assays measuring pre-existing AAV immunity, given the vast diversity of methods and technologies used across companies and academic institutions. However, with the advent of commercially available AAV companion diagnostics (CDx) assays developed and validated by third parties, there may be a potential non-competitive situation on the horizon where various different drug developers could resort to using the same CDx kit. The first commercial CDx kit for detecting AAV5 antibodies (from ARUP, to be used for BioMarin's Hemophilia A Gene Therapy) is currently undergoing final regulatory review and is expected to be approved and marketed later this year.

Another possible next step towards standardization of assays to measure pre-existing AAV immunity may be to establish a publicly available reference set of highly purified, monoclonal human anti-AAV antibodies (i.e. positive control reagents), separately for each AAV serotype. A similar approach has been used previously for the methods designed to assess neutralizing antibodies directed against EPO [Mytych et al 2012]. For example, the first WHO standard consisted of a panel of anti-EPO antibodies, including pre-existing non-neutralizing antibodies, treatment-induced early onset antibodies, and affinity-matured highly neutralizing antibodies. These antibodies were used in TAb or cell-based NAb assay formats across different laboratories (industry, academia, CRO) to compare method performance and confirm detectability and titer for the different types of antibodies ((Wadhwa et al 2016). They were tested alongside real clinical samples to ensure commutability and to test that the monoclonal antibodies behaved in a similar way to a patient sample.

Resolving the numerous hurdles encountered in each of these cases meant that despite the strong commitment and high engagement of different participants, the process was lengthy and time-consuming. Most importantly, participation from various stakeholders including regulators, clinicians, wider industry was necessary for a successful outcome. In the context of standardizing measurements for anti-AAV immunity, which as stated previously has increased complexities, can any or both these approaches be adopted or is there another alternative that could potentially be considered? While a brief discussion of pros and cons of these options is presented in this article, the decision on adoption of either of these routes will ultimately require consensus, continual engagement and commitment/participation from various stakeholders including regulators, clinicians, companion diagnostics (CDx) companies and a wider industry (academia?? others).

If applying this approach to pre-existing AAV immunity, the first challenge would be to identify and isolate anti-AAV antibody coding sequences, preferably from healthy human donors. The monoclonal antibodies would then need to be produced by a central repository in sufficient quantity and quality, followed by distribution to various local testing laboratories for a comparative field study. It may be possible to utilise existing monoclonal antibodies described in the literature (Gurda, 2012; Harbison, 2011; Moskalenko, 2000) and parallel sequencing that highlights capsid structure and function combined to gain an overall perspective of the immunological response to AAVs (Adachi, 2014).

Using reference panels for a broader level of characterization of assay sensitivity may be of interest not only to companies developing AAV gene therapies in clinical studies but also to companies developing AAV companion diagnostic (CDx) kits for commercial use, after AAV gene therapies have been approved for marketing. As more clinical experience is gained with commercially available CDx kits, a future regulatory guidance could aim to establish and specify minimally required sensitivity for detecting AAV antibodies from the reference panel to ensure the predictability of desired clinical outcomes based on the CDx results.

A viable alternative or complementary reagent to purified human monoclonal anti-AAV antibodies could be a **reference panel with polyclonal human anti-AAV antibodies in crude human plasma from individuals who were tested positive**; this may need to be pooled across multiple donors to achieve a sufficient quantity of the reference material, to provide a more homogeneous standard, and to be representative of the full repertoire of antibody response. While this strategy may be easier to accomplish in the short term, it leads to difficulties in maintaining lot-to-lot consistency in the long term. In any case, such a panel of samples may be useful to include even for the collaborative study in which the isolated monoclonal AAV antibodies are tested.

Instead of establishing human monoclonal anti-AAV antibody reference panels, a more timely and cost-effective approach could be to harmonize different assay methodologies by performing proficiency studies across laboratories using aliquots of the same test sample. This proficiency test samples could either be an AAV-reactive human plasma sample or negative human plasma sample spiked with varying levels of a non-human monoclonal positive-control antibody. The aliquots are shared across laboratories and test results are reported. The disadvantage would be the limited longitudinal availability of the reference sample, due to finite volumes of human plasma samples and due to potential future manufacturing changes or supply shortages in the non-human positive-control antibody reagent.

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Terminology (definitions)

AAV - adeno-associated virus – AAVs are members of the parvovirus family, which are non-enveloped, encapsidated viruses that have a single-stranded DNA genome. They are small viruses of approximately 20-25 nm in diameter that require helper viruses for replication. They were first discovered in the 1960's as a contaminant of adenovirus stocks.

Serotype - The AAVs are classified into serotypes according to their genome sequence similarities. Serotypes 2,3,5 and 6 were discovered in human adenovirus preparations whilst 1,4, 7-11 were isolated from non-human primates.

Antibody Titer – Antibody titer is defined as the reciprocal of the highest dilution of a test sample that is detected as positive using a particular assay. Titer values are contingent upon the sensitivity of the assay used and cannot be directly compared between assays with different sensitivity.

End point (Standard) Titer – determined based on highest empirically observed test sample dilution that was detected as positive

Extrapolated Titer – determined based on the highest theoretically achievable test sample dilution that would be detected as positive at the cutpoint of the assay

Total Antibodies -

Assay Types

Neutralizing Antibodies (NAb) – Antibodies that circulate in the blood and are able to bind their specific target. Neutralizing antibodies, when bound, have a negative impact on function.

Total Binding Antibodies (TAb) - Antibodies that circulate in the blood and are able to bind their specific target, inclusive of neutralizing antibodies and antibodies that do not have negative impacts on function.

ELISA – Enzyme linked immunosorbent assay, a test to measure the amount of antibody binding a protein target.

Cell based Antibody Assay – A test that requires living cells in culture to measure the capacity of antibodies to neutralize the function of its binding target, such as virus neutralization assay.

Negative control – A sample that does not contain AAV antibodies and thus remains negative in the assay.

Positive control - A sample that contains AAV antibodies and is detected as positive in the assay.

Titer control – A positive control that contains a particular quantity of AAV antibodies and is measured within a pre-specified titer range.

Titer value - the value is obtained by testing serial dilutions of a sample in the assay and determining the highest sample dilution that still generates a positive response. The titer is then equivalent to the reciprocal of that sample dilution (e.g., a sample dilution of 1:1000 corresponds to a titer of 1000).

Pre-existing immunity – Previous infection or therapy with the same or similar AAV serotype can lead to recognition of a subsequent infection or gene therapy by the immune memory response, thereby reducing or even preventing the second infection or therapy.

Standard (documentary and physical) – Standards can be documents that offer guidance or legal requirements that must be followed, physical standards are materials that can be used in laboratories to develop and standardize tests.

IS - The highest order of standard is an International Standard (IS) that has been endorsed by the World Health Organization and is used globally to calibrate assays. A secondary standard is a physical standard that has been calibrated to the IS.

Regulatory Guidance (FDA etc.) Official set of rules published by legal health authorities that govern certain aspects of drug development, such as development and validation of immunogenicity assays

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