Evaluation of Immune Responses to Novel Adeno-Associated Viruses for Vaccine and Gene Therapy Applications

by

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ABSTRACT

The transfer of a desired gene to several types of target tissues has been accomplished successfully in the past using existing Adeno-associated viruses (AAVs). Also, it has recently been shown that AAV can stimulate robust antibody responses due to long-term transgene expression or abolishment of transgene product by cell-mediated immune responses, suggesting the potential use of AAVs as vaccines. Most humans already have pre-existing immunity to common AAV serotypes making novel AAVs of low seroprevalence attractive as gene transfer or vaccine vehicles. This thesis describes my primary research objectives that included the isolation of novel AAV serotypes based on AAV DNA sequences from porcine tissues, novel AAV vector production, and biological characterization of porcine AAVs *in vitro* and *in vivo*. This was followed by evaluating immune responses in mice vaccinated with porcine AAV vectors expressing the hemagglutinin (HA) from the avian influenza A/Hanoi/30408/2005 (H5N1) strain. These findings show that low seroprevalence porcine AAV vectors were able to efficiently transduce a wide range of cells and tissues. The porcine vectors also performed well as vaccine candidates and were efficient at stimulating host immune responses. Although porcine vectors were successful as vaccines, further studies involving long term gene expression by porcine AAVs is still necessary to confirm their role as gene therapy vehicles.

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Table of Contents

ABSTRACT II

ACKNOWLEDGMENTS III

TABLE OF CONTENTS V

LIST OF TABLES VII

LIST OF FIGURES VIII

LIST OF ABBREVIATIONS IX

1.0 – ADENO-ASSOCIATED VIRUS 1

1.1 – Introduction to AAV 1
1.2 – Taxonomy and Classification of AAV 2
1.3 – Biology of AAV 5
1.4 – General Properties of AAV 7
1.5 – Structure and Function of AAV Capsids 7
1.6 – Cellular Binding and Entry of AAV 9
1.7 – Intracellular Trafficking of AAV and Travel to the Nucleus 10
1.8 – Structural Organization of AAV Genome 12
1.9 – Release and Replication of AAV Genome in the Nucleus 15
1.10 – Immune Responses to AAV 16
1.11 – Pre-existing Immunity to AAV in Humans 18

2.0 – RECOMBINANT AAV VECTORS 19

2.1 – Introduction to AAV Vectors 19
2.2 – Production of Recombinant AAV Vectors 19
2.3 – AAV Vectors for Gene Therapy 22
2.4 – AAV Vectors and Immune Evasion 25
2.5 – AAV as a Vaccine Vector 27
2.6 – Risks Associated with the Use of AAV Vectors 28

3.0 – INFLUENZA A VIRUSES 29

3.1 – Overview and General Properties of Influenza A Viruses 29
3.2 – Avian Influenza Viruses 30
3.3 – Prevention of an Avian Influenza Pandemic 31

4.0 – OVERVIEW OF OBJECTIVES AND STATEMENT OF HYPOTHESIS 32

5.0 – MATERIALS AND METHODS 34

5.1 – Isolation of Genomic DNA from Porcine Tissues and Amplification of AAV Sequences 34
5.2 – Cloning Procedures for PCR Products Involved with AAV Vector Production 39
5.3 – Hybrid Porcine AAV Particle Production and Purification 41
5.3.1 – Plasmids Involved with Production of Hybrid Porcine AAV Particles *41*
5.3.2 – Cell Culture and DNA Transfections *42*
5.3.3 – Purification of AAV Particles *44*
5.3.4 – Cesium Chloride Gradient Purification of AAV Particles *45*
5.3.5 – Fractionation of Cesium Chloride Gradients *45*
5.3.6 – Titering of AAV Particles by Real Time PCR *46*
5.4 – Measurement of Protein Expression by Hybrid AAV Vectors 49

5.5 – Electron Microscopy of Produced Hybrid AAV Particles 50
5.6 – Transduction Efficiency of Novel AAV Vectors *In Vitro* 51
5.7 – In Vivo Studies Involving AAV Vectors expressing *lacZ* 51
5.7.1 – Immunization Schedule 51
5.7.2 – Biodistribution of Novel AAV Vectors 53
5.7.3 – Histological Analysis of Novel AAV Vectors 53
5.7.4 – Serology of Novel AAV Vectors 54
5.8 – In Vivo Studies Involving AAV Vectors expressing HA 55
5.8.1 – Immunization and Challenge Schedule 55
5.8.2 – Characterization of T Cell Responses to Novel AAVs 55
5.8.3 – Characterization of B Cell Responses to Novel AAVs 57
5.9 – Animal Containment and Scoring During Studies 58

6.0 – RESULTS 60

6.1 – Identification of Novel AAV Sequences from Porcine Tissues via PCR 60
6.2 – Production of Hybrid Porcine AAV Vectors Expressing LacZ 62
6.3 – Transduction Efficiency of Hybrid Porcine AAV Vectors *In Vitro* 64
6.4 – Biodistribution and Histology of Porcine AAV Vectors Expressing LacZ 65
6.5 – Cross Neutralization and Seroprevalence of Novel AAV Vectors 67
6.6 – Production of Hybrid Porcine AAV Vectors Expressing H5N1-HA 68
6.7 – Characterization of T Cell Responses to Novel AAVs *In Vitro* 69
6.7.1 – T Cell Response Kinetics to Novel AAVs 69
6.7.2 – Protection Studies of Novel AAV Vectors Following Lethal Challenge 70
6.8 - Characterization of B Cell Responses to Novel AAVs *In Vitro* 72
6.8.1 – Antibody Responses to Novel AAVs 72

7.0 – DISCUSSION 74

7.1 – Isolation and Production of Novel Porcine AAV Serotypes 74
7.2 – Transduction Efficiency and Tropisms of Novel AAV Vectors Expressing *lacZ* 75
7.3 – Generation of Porcine H5N1-HA AAV Vectors for Immunological Studies 77
7.4 – Selection of *In Vivo* Model and Dose for Immune Characterization of AAV 79
7.5 – *In Vivo* T Cell Responses to Porcine AAVs Expressing HA 80
7.6 – *In Vivo* B Cell Responses to Porcine AAVs Expressing HA 81
7.7 – Protection Studies of Porcine AAVs Following Lethal Challenge in Mice 82

8.0 – FINAL THOUGHTS AND FUTURE DIRECTIONS 83

List of Tables

Table 1.6 – Cell Receptors and Target Tissues Identified for AAV Serotypes

Table 2.3 – Overview of rAAV Vectors Used in Clinical Trials

Table 5.1 – Nested TAIL PCR Protocol for Isolation of AAV *cap* Gene

Table 5.3 – RT-PCR Reaction Mixture for Titering of Hybrid AAV

Table 6.1 – AAV Serotype Percent Identities Based on Sequence Homology

Table 6.5 – Cross-Neutralization and Seroprevalence of Porcine AAVs

List of Figures

Figure 1.2 – Organization of the Family Parvoviridae

Figure 1.8 – Schematic Representation of AAV Genome

Figure 2.2 – Production of Recombinant AAV Vectors

Figure 5.1 – Overview of Isolation of Novel Porcine AAVs

Figure 6.1 – Phylogenetic Tree Comparison of Novel Porcine AAVs

Figure 6.2a - Western blots of Porcine AAV Capsid Proteins

Figure 6.4 – Histology of Porcine AAV Vectors in BALB/c Mice

Figure 6.6 - Western blots of H5N1 expression from porcine AAVs

Figure 6.71 - Pooled H5N1-HA Peptide Responses for IFN-γ at Day 40

Figure 6.72 – Protection of Vaccinated Mice Challenged with Hanoi 2005

Figure 6.81 – HI titres against Hanoi 2005 in BALB/c Mice

List of Abbreviations

AAV: Adeno-associated Virus

AD: Hemeagglutinating Doses

APC: Antigen Presenting Cells

BGH: Bovine Growth Hormone

bp: Base Pair

CsCl: Cesium Chloride

d.p.i.: Days Post Injection

DLP: Dual Labelled Probe

DOC: Deoxycolate

ELISpot: Enzyme-Linked Immunosorbent Spot

FACS: Fluorescent Activated Cell Sorting

FGFR1: Fibroblast Growth Factor Receptor 1

GC: Genome Copies

HI: Hemagglutination Inhibition

HSPG: Heparan Sulphate Proteoglycans

IM: Intramuscular

IN: Intranasal

ITR: Inverted Terminal Repeat

IV: Intravenous

LB: Lysogeny Broth

MHC: Major histocompatibility complex

NAbs: Neutralizing Antibodies

NHP: Non-Human Primate

NLS: Nuclear Localization Signals

NML: National Microbiology Laboratory

NPC: Nuclear Pore Complex

ORF: Open Reading Frame

PBS: Phosphate Buffered Saline

PLA_2: Phospholipase A_2

RBE: Rep Binding Element

RFM: Replicating Form Monomer

RFD: Replicating Form Dimer

RDE: Receptor Destroying Enzyme

RT-PCR: Real Time Polymerase Chain Reaction

SFU: Spot Forming Units

ssDNA: single-stranded DNA

TAIL: Thermal Asymmetric Interlaced

TE: Transduction Efficiency

Treg: Regulatory T-Cell

TV: Tail Vein

wt: Wild Type

1.0 – Adeno-associated Virus

1.1 – Introduction to AAV

Adeno-associated Virus (AAV) belongs to the family *Parvoviridae* which are among the smallest known viruses and the genus *Dependovirus*. AAV is a small, nonenveloped virus that is 25nm in diameter containing a linear, single-stranded DNA (ssDNA) genome approximately 4.7 kb in size. AAV replication is dependent on co-infection with an unrelated helper virus, generally an adenovirus (Ad) or herpesvirus, but possibly with a papillomavirus as well [1].

AAV was discovered during 1960-1966 when it was first thought to be a contaminant of Ad preparations. Upon closer inspection via electron microscopy it was observed that there were in fact two distinct particles, a larger Ad particle and a smaller particle that was reported to be a second virus. It was noted that this smaller virus depended on coinfection with Ad for productive infection, and therefore was named adeno-associated virus. Subsequently, a number of herpesviruses were also shown to serve as helpers for AAV replication [2].

In the past, AAV was not thought to be of great medical significance due to its non-pathogenic nature. As a result, the lack of general knowledge about this virus initially hindered its broad use as a viral vector. As research in the AAV field advanced, so did the number of AAV serotypes that were discovered. Thus far, twelve human serotypes of AAV and more than 100 serotypes from nonhuman primates (NHPs) have been

discovered. The lack of known pathogenicity in humans, combined with the ability of viral persistence and the many available serotypes have increased the potential use of AAV as a delivery vehicle for use in gene therapy as well as vaccine applications [3]. Human AAV vectors are among the most commonly used viral vectors for gene therapy today and have successfully been utilized to promote sustained gene expression in a variety of tissues including muscle, eye, brain, liver, and lung [4]. It should be noted that AAV vectors are also currently being evaluated as vaccine platforms for various diseases such as Influenza virus and Human Immunodeficiency Virus (HIV) [5].

1.2 – Taxonomy and Classification of AAV

AAV belongs to the family *Parvoviridae* which encompasses viruses that are nonenveloped and contain icosahedral capsids ranging from 18-26 nm in diameter. The capsids contain a linear ssDNA molecule that can range between 4 and 6 kb in length. The family *Parvovirdae* is separated into two subfamilies consisting of the *Parvovirinae* that infect vertebrates and the *Densovirinae* which infect insects along with other invertebrates. The *Parvovirinae* are further divided into five genera: the parvoviruses, erythroviruses, amdoviruses, betaparvoviruses and dependoviruses. The *Densovirinae* are also further divided, but into four genera: the densoviruses, iteraviruses, pefudensoviruses and brevidensoviruses [6] (Figure 1.2).

Members of the *Parvovirinae* are classified by their biological and genomic properties. For the sake of simplicity only dependoviruses will be discussed. Dependoviruses

contain capsids with equal amounts of both negative and positive sense ssDNA that are linear and approximately 4.7 kb in size. The genome is structurally characterized by 145bp inverted terminal repeats (ITRs) at each end of the AAV genome which flank two open reading frames (ORFs) encoding the viral replication (*rep*) and capsid (*cap*) genes. The first 125bp of each ITR forms a palindrome which folds upon itself to maximize base pairing and forms a T-shaped hairpin structure. There are also three mRNA promoters and a single polyadenylation site near the 3′ end. Dependoviruses were given their name due to the fact that their life cycle is "dependent" on the presence of a helper virus for efficient viral replication, although AAV replication has been observed in the absence of helper viruses under specific conditions [7]. Of the recognized AAV serotypes thus far, AAV2 has been the most broadly studied, and therefore the following discussion of AAV biology will focus primarily on this serotype.

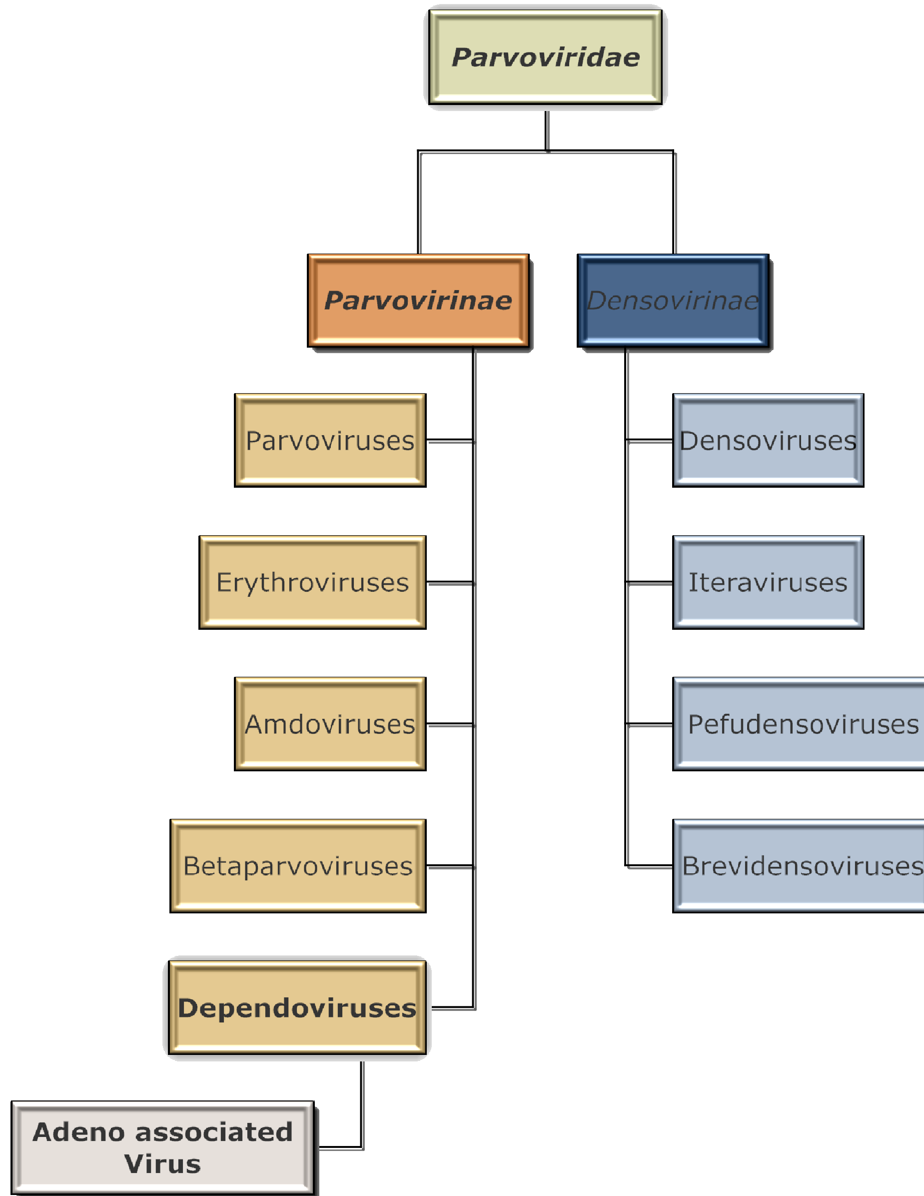


Figure 1.2 – Organization of the Family Parvoviridae
Tree diagram depicting the members of the family *Parvoviridae*. AAV belongs to the genus *Dependovirus* under the *Parvovirinae* sub-family.

1.3 – Biology of AAV

Adeno-associated viruses (AAVs) contain a small icosahedral capsid that is structurally stable and encompasses a small genome with few genes to control their host interactions. AAV binds to specific host cell receptors and only replicates in dividing cells in the presence of a helper virus. The host and tissue tropism of AAV is based primarily on the capsid structure and its ability to bind and use host cell receptors. AAV has not been shown to cause disease and although AAV is restricted by its dependence on helper viruses for replication, it still has a broad host range as exemplified by seroprevalence in many natural hosts including humans, monkeys, horses, cows, sheep and birds. AAV can be spread among hosts by an assortment of routes, including fecal-oral, urine, and respiratory spread or by contaminated fomites in the environment. Although the routes of viral entry into the body have not been clearly illustrated experimentally, it is believed that AAV uptake happens via dividing cells of the upper respiratory tract, oropharynx, intestine, or by infecting epithelial cells and local lymphoid tissue which contains dividing cells [8].

The ability of AAV to integrate its DNA into the host cell and start a latent infection is thought to account for its persistence in nature [9]. When AAV infection of a healthy cell occurs without the presence of a helper virus, the viral genome fails to replicate and instead initiates a persistent infection via chromosomal integration. It still remains unclear whether AAV commonly initiates a latent infection via integration in host cells [10]. The AAV genome frequently form concatamers, which are long continuous DNA

molecules containing multiple copies of the same DNA sequences linked in series (usually a result of Rolling Circle Replication) [11]. These concatamers that are formed then remain in an extrachromosomal state. The incorporated AAV genome has the ability to be rescued *in vitro* through superinfection with a helper virus. It should be noted that recombinant AAV (rAAV) does not routinely integrate its DNA into host cells; instead it persists in the nucleus episomally and forms concatamers [12]. The ability of AAV to persist in an extrachromosomal or integrated state can be seen as an effective way for persistence if the infected cells are not actively dividing. Nonetheless, in dividing cells, any extrachromosomal AAV would most likely be diluted out during cellular replication. It has also been observed that a number of factors can stress host cells resulting in changes to the intracellular environment that permit AAV replication to take place. *In vitro*, such factors are mainly associated with genotoxic conditions (e.g. ultraviolet irradiation, ionizing radiation, cycloheximide, etc) [13].

The relationship between AAV and its respective helper viruses is fairly versatile. AAV has the ability to hinder the replication of Ad based on comparative amounts of the two viruses and the status of the coinfection [14]. When the relative amount of AAV to Ad is too high, AAV viral replication proteins can control Ad gene expression and prevent the replication of both viruses. Alternatively if the relative amount is lower, AAV replication continues while Ad replication is still repressed. When Ad infection initially occurs and DNA replication has already begun, any AAV infections taking place are productive and Ad replication is not inhibited. The AAV Rep protein functions to inhibit heterologous

promoters, and the same phenomenon is seen in coinfections of AAV with other viruses such as papillomaviruses and herpesviruses [15] [16]. It still remains unknown to what degree the inhibitory effects of AAV infection have on the host *in vitro*.

1.4 – General Properties of AAV

The molecular weight of an AAV particle can range from 5.5 to 6.2 × 10^6 Da and half of this weight is made up of protein with the remainder consisting of DNA. AAV virions have a buoyant density of 1.39 to 1.42g/cm^3 in cesium chloride (CsCl) [17], and their sedimentation coefficient in neutral sucrose gradients ranges from 110 to 122 [18]. AAV virions are quite resilient, remaining stable at a pH range from 3 to 9 and at a temperature of 56°C for 1 hour. However, AAV can be inactivated by hydroxylamine, formalin, β-propriolactone and oxidizing agents. AAVs properties of being robust and also easy to isolate in CsCl gradients make these viruses favourable for therapeutic uses.

1.5 – Structure and Function of AAV Capsids

AAV capsids are made up of 60 protein subunits and have a triangulation number of one (T=1), this is also the smallest number of subunits required to construct an icosahedral capsid. As a result, AAVs are structurally amongst the simplest viruses that exhibit icosahedral symmetry. Conversely, instead of being made up of a single type of structural protein, its subunits are constructed from 3 different structural proteins, including VP1 (90 kDa), VP2 (72 kDa), and VP3 (60 kDa). The structural proteins of AAV are present at a ratio of about 1:1:10 with VP3 making up close to 90% of the

capsid [19]. It should be noted that these structural proteins are interrelated because they are encoded by in-frame, overlapping gene sequences. Therefore, the complete coding sequence for VP3 is found within the coding sequence for VP2, and the coding sequence for VP2 is found within the coding sequence for VP1. All 3 capsid proteins share the same C-terminal domain given that they are translated in the same reading frame and this domain is thought to be necessary for cell binding [20].

The unique N-terminus of the VP1 minor coat protein contains a calcium dependent lipolytic enzyme designated as phospholipase A_2 (PLA_2). PLA_2 is normally sequestered within the capsid structure but becomes exposed during cellular entry, which allows for viral escape from the endosomal compartment into the cytoplasm. The N-terminus of VP1 also contains basic amino acid motifs that play a role as nuclear localization motifs during cellular entry and capsid assembly [21].

The core of the C-terminal domain consists of an eight-stranded, antiparallel β-barrel motif and most of the AAV capsid structure is made up of large loops that are inserted between the β strands of the β-barrel. The loops are responsible in the formation of projections at the icosahedral threefold axes and also the depressions at the twofold and around the fivefold axes. In addition to the structural formations of AAV capsids, these loops account for many other functions which include host and tissue tropisms, receptor binding and contain epitopes recognized by host immune responses for antibody binding [22].

1.6 – Cellular Binding and Entry of AAV

The process by which AAV enters a cell is dependent on its specific cellular tropism as well as the host itself due to cell receptor variation. AAV encompasses many serotypes which can have a number of different tropisms and infect a range of hosts resulting in the use of varying receptors for cellular binding. Infection of a cell with AAV can involve the participation of more than one cell surface molecule, usually a primary receptor followed by secondary or coreceptor. Several AAV serotypes bind to heparan sulphate proteoglycans (HSPG) on the cell surface and utilize these molecules as a low-affinity primary receptor. After binding HSPG, some serotypes then employ integrins as their secondary receptor, while others may use the fibroblast growth factor receptor 1 (FGFR1) for this function. There are also other receptors and coreceptors that have been shown to be used by AAV which includes N-linked sialic acid, O-linked sialic acid, laminin and platelet-derived growth factor receptor (PDGFR) [23] (Table 1.6). Although AAV particles attach to a primary receptor on the host cell, their entry is dependent on subsequent interaction of the particle with the secondary or co-receptor. This interaction is known to prompt conformational changes in the AAV particle that allow for entry into the host cell. Following receptor binding, AAVs enter into the cell via receptor-mediated endocytosis and some AAV serotypes have been observed using clathrin-coated vesicles to enter target cells [24].

Table 1.6 – Cell Receptors and Target Tissues Identified for AAV Serotypes

AAV Serotype	Receptor Used	Target Tissue
AAV1	N-linked sialic acid	Muscle, Liver, Joint, Heart
AAV2	HSPG αVβ5 integrin FGFR1 Laminin	Lung, Muscle, CNS, Liver, Joint, Eye
AAV4	O-linked sialic acid	CNS, Eye
AAV5	N-Linked sialic acid PDGFR	CNS, Eye, Liver, Lung
AAV6	N-Linked sialic acid	Muscle, Heart, Lung, Liver
AAV7	Unknown	Muscle, Liver
AAV8	Laminin	Muscle, Liver, Eye
AAV9	Laminin	Liver, Lung, Heart
AAVrh.10	Unknown	Lung, CNS
AAV2/5	HSPG	Muscle

Abbreviations: HSPG, heparan sulphate proteoglycan; FGFR1, fibroblast growth factor receptor 1; PDGFR, platelet-derived growth factor receptor.

1.7 – Intracellular Trafficking of AAV and Travel to the Nucleus

Once inside the cell, AAV particles must escape from their endosomal compartment in order to travel to the nucleus. It should be noted that because AAV replicates in the nucleus it is required to cross two membranes, first the endosomal/lysosomal compartment, then the nuclear membrane. AAV is non-enveloped and therefore does not

have the same ability as enveloped viruses that can enter the cytoplasm through fusion of their viral envelope with a specific membrane. The mechanism by which AAV escapes from the endosomal compartment and gains entry into the cytoplasm is poorly understood [25]. Previous studies have shown that acidification of the endosomal compartment is critical for escape and successful infection [24]. More recent findings illustrate that AAV may use an enzymatic process to break through and cross the endosomal membrane. This process involves the use of the lipolytic enzyme PLA_2 that is expressed on the N-terminus of the VP1 minor coat protein of AAV, which is vital for infection and increases capsid release into the cytoplasm [25]. This enzymatic region of VP1 is normally contained inside the capsid structure but is later exposed in the late endosomal compartment, thought to be caused by a low pH. AAV capsids are most likely released from the endosome into the cytoplasm at a perinuclear location. In the cytoplasm, additional processing and trafficking events probably occur prior to nuclear transport [24].

The N-terminus of VP1 encompasses three nuclear localization signals (NLSs) that are revealed in conjunction with the exposure of the PLA_2 catalytic domain. Two of these NLSs are also present at the N-terminus of VP2 and thus also exposed in the late endosome. The AAV particles that escape from the endosome undergo conformational changes and their molecular organization remains unclear. However, it is known that the AAV genome must be transported to the nucleus and this process involves the use of a nuclear import pathway [26]. Nuclear import requires that the virion entering the nucleus

express a NLS and these are expressed on both the VP1 and VP2 of AAV. Nuclear entry of AAV is thought to engage the nuclear pore complex (NPC) even though this has not been decisively proven. Studies involving other parvoviruses have demonstrated that viral capsids affect the structure of the nuclear envelope at or near the NPC [27]. Other studies also propose that AAV may actually travel through the nuclear pore with the capsid intact due to its very small size. Evidence to support this was found during an experiment where several capsid-specific antibodies were microinjected into the cell nucleus and blocked AAV2 infection completely [28]. The ultimate destination of AAVs is in the nucleus, and whether the viral particles enter the nucleus intact or as naked DNA remains unclear.

1.8 – Structural Organization of AAV Genome

AAV contains a linear, ssDNA genome approximately 4.7 kb in size [29]. AAV genomes can consist of either a positive (+) sense or negative (-) sense DNA strand which are packaged into the AAV capsid with the same frequency. The genome is structurally characterized by 145bp ITRs at both the 5′ and 3′ ends, which flank two ORFs that encode the AAV *rep* and *cap* genes. The genome also includes three promoters for the initiation of transcription, P5, P19, P40 and contains a single polyadenylation site (Figure 1.8). The first 125bp of the ITR comprises a palindrome that folds upon itself to enhance base pairing. Base pairing of the palindromic sequences results in the formation of a T-shaped hairpin structure whereas the remaining 20bp, designated as the “D sequence” are left unpaired [30]. The ITRs are functionally

important *cis*-active sequences that have a key role in the replication of AAV DNA.

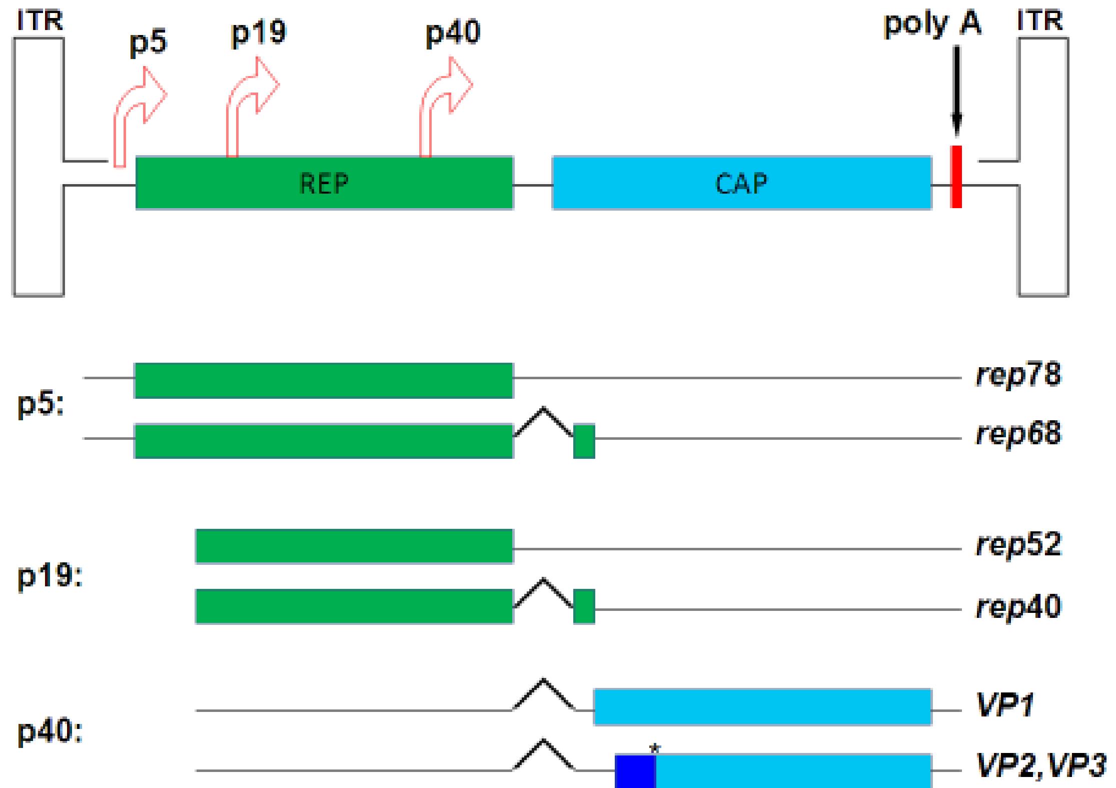


Figure 1.8 – Schematic Representation of AAV Genome
Diagram illustrating organization of the AAV genome. It is made up of two ORF consisting of *rep* and *cap* genes that are flanked by ITRs. The different Rep and Cap transcripts are produced by their respective promoters made up of p5, p19, and p40. Asterisk indicates position of alternative start codon ACG required for production of VP2.

With the current understanding of AAV replication, the ITR functions as the origin of replication and acts as a primer for second-strand synthesis by DNA polymerase. The dsDNA that is produced during the second-strand synthesis is called the replicating form monomer (RFM). The RFM is used for another round of self-priming replication and forms a replicating form dimer (RFD). The dsDNA intermediates (RFM and RFD) are

modified by a strand displacement mechanism that results in dsDNA used for transcription and ssDNA used for packaging [26].

The Rep binding elements (RBEs) RBE and RBE$^{/}$, along with a terminal resolution site (TRS), both located within the ITR, are vital to the AAV replication process. The AAV Rep protein uses the RBEs and TRS during replication to further develop the dsDNA intermediates. Further to their role in AAV replication, the ITRs are also important for AAV transcription, negative regulation of viral replication during unfavourable environmental conditions, genome packaging and site-specific integration.

The first ORF within the AAV genome contains the *rep* gene which generates four Rep proteins, Rep78 and Rep68 (involved in replication) as well as Rep52 and Rep40 (involved in encapsidation) (Figure 1.8). The larger Rep78 and Rep68 proteins are produced from transcripts using the P5 promoter, while the smaller Rep52 and Rep40 proteins are produced from transcripts using the P19 promoter. The Rep78 and Rep68 are central proteins involved in regulation that are generated from spliced and unspliced transcripts operating in trans during all phases of the AAV life cycle. These proteins are responsible for positive and negative regulation of AAV gene expression in the presence or absence of a helper virus and are needed for DNA replication. Rep52 and Rep40 are involved in the accumulation of viral ssDNA used for packaging into AAV capsids, and are also produced from spliced and unspliced transcripts. It should be noted that all four Rep proteins have helicase and ATPase activity [27]. The larger Rep proteins take it a

step further by exhibiting site-specific DNA binding activity (occurs at the RBE) and also display site- and strand-specific endonuclease activity (nicking the TRS).

The second ORF inside the AAV genome contains the *cap* gene, responsible for generating the three capsid proteins of AAV (VP1, VP2, and VP3). All of the capsid proteins are produced from alternative splicing of two transcripts via the P40 promoter. The largest capsid protein VP1 (87kDa) is produced from the unspliced transcript and the remaining capsid proteins VP2 (72 kDa) and VP3 (62 kDa) are produced from the spliced transcript. An alternative ACG start codon is used to make VP2, while VP3 is generated downstream via the conventional AUG start codon [28].

1.9 – Release and Replication of AAV Genome in the Nucleus

As mentioned previously, it remains unclear as to how the ssDNA genome of AAV is released from the capsid for replication. One explanation is that AAV may have 20 to 30 nucleotides of the 5′ end of its genome exposed on the outside of the capsid with the Rep protein covalently attached to it [31]. Another explanation is that the 3′ end of the AAV genome can also become exposed outside of the capsid exclusive of capsid degeneration [32]. As a result, this exposed DNA could function as a template for initiating DNA replication using the host cell DNA polymerase within the nucleus. In this case the DNA would be separated from the capsid without breakdown of the capsid. Alternate methods for release of the DNA from the AAV capsid, including breakdown of the capsid are possible but are currently unknown.

Replication for all AAVs takes place in the nucleus and depends on cellular enzymes along with the production of dsDNA replicative intermediates that have the dual purpose of also serving as templates for transcription. The cellular enzymes required for DNA metabolism are only efficiently generated in cells that are in S phase of the cell cycle. Therefore AAV is dependent on helper viruses such as Ad that induce "resting" cells to enter into S phase which allows for replication to proceed. Since AAVs possess multiple Rep proteins that have various functions in respect to DNA replication, but lack a DNA polymerase or ssDNA binding proteins, additional factors are supplied by the host cell, a helper virus or both.

1.10 – Immune Responses to AAV

In humans, pre-existing immunity to wild type (wt) AAV2 is largely humoral, with a small population of people showing minor IL-10 secretion and lymphocyte propagation in response to AAV2 proteins [33]. Immune responses in immunocompetent mice and rhesus macaques proved to be similar as well, displaying activation of helper T-cell (Th2) subsets and B-cells to viral capsid proteins one year post vaccination with AAV2. These experiments also showed AAV2-specific IgM and IgG2 responses, signifying that host immune responses relied upon both T cell-dependent and -independent mechanisms [34].

In a study where wt AAV2 was used as an infectious model in primates, rhesus macaques showed that primary and memory immune responses were dependent upon the route of

infection and the presence of helper virus. Intravenous (IV) and intramuscular (IM) routes of infection with AAV2 were able to elicit neutralizing antibodies (NAbs) on their own whereas intranasal (IN) infection was unable to elicit the same response. On the contrary, IN co-infection of AAV2 with Ad elicited NAbs and lymphocyte proliferative responses to AAV2 along with cellular infiltration in local tissue [35]. It should be noted that these findings most likely resulted from a number of factors including: a potential immune adjuvant effect of Ad coinfection, host exposure to higher doses of AAV2 proteins due to active viral replication, and systemic AAV2 spread due to disruption of the mucosal barrier by Ad infection [34].

Murine models showed that the level of the host immune response to AAV2 was dependent on the viral dose and route of infection. This correlated with the different threshold values required for the generation of NAbs in C57BL/6 mice in different tissues. In the mouse lung model the value for significant NAb production was 10^6 AAV2 particles per mouse when injected via the intrapulmonary route, whereas this value was higher in the muscle at 10^8 AAV2 particles per mouse when injected IM [36, 37]. Results also showed that neutralizing responses actions against IM or IV injected AAV2 in mice was entirely T cell dependent, since mice deficient in functional T-cells (BALB/c or C57BL/6 nude mice, CD40L knockout mice and $CD4^+$ T cell depleted mice) did not produce NAbs, even though they produced comparable amounts of IgM after immunization. Nevertheless, AAV2 delivered to the liver of mice or rhesus monkeys formed short-lived neutralizing humoral immune responses that were T-cell independent

[36].

1.11 – Pre-existing Immunity to AAV in Humans

In humans, the frequency of serum antibodies specific to AAV1, 2, 3 and 5 is quite widespread. Previous seroepidemiological studies with AAV1, 2, 3 and 4 showed that the incidence of seroprevalent antibodies against AAV1, 2 and 3, increased sharply between the ages of 1 and 10, reaching a peak of 60% by the age of 10. Antibodies against AAV4, initially isolated from non-human primates (NHPs) was found to be much less frequent, having a peak frequency of 10% between the ages of 2 and 5 years [38, 39]. A more recent study involving the epidemiology of AAV2 showed that seroprevalence increased with age and was dispersed worldwide. They found that 30% of individuals less than 10 years of age were seropositive for AAV2, which grew to 60% in persons between 10 and 19, decreased to 49% in persons between 20 and 29, then increased after age 30, reaching a peak of 73% in persons 50 to 59 years of age [40].

These findings are important because they demonstrate that the presence of NAbs to common AAV serotypes is widespread with AAV2 being the most common AAV to infect humans. NAbs to AAV can hinder *in vivo* transduction of cells and tissues subsequent to systemic administration of rAAV, rendering gene therapy and vaccine applications with these AAV serotypes to be unsuccessful [33]. For these reasons, a number of approaches to overcome pre-existing AAV immunity are being pursued and will be discussed in the following chapters.

2.0 – Recombinant AAV Vectors

2.1 – Introduction to AAV Vectors

Early studies of AAV focused on understanding the biology of AAV which was conducted primarily with human AAV2. Since that time, there has been an astounding increase in the amount of AAV research motivated by two major fields: the biology of AAV and the development of AAV vectors. This second field has become quite popular due to the initial evidence provided by AAV gene expression studies and also the safety associated with AAV vectors. As a result, this field has attracted a great amount of investigators keen on developing AAV vectors for therapeutic applications. At the same time, the development of these vectors greatly increased research into AAV biology and in turn supplied more refined tools to augment these investigations, making the two fields completely entwined.

2.2 – Production of Recombinant AAV Vectors

Recombinant AAV (rAAV) vectors are produced in such a way that only the ITRs are retained as *cis*-active DNA elements required for rAAV vector replication and packaging. The AAV coding regions for the *rep* and *cap* genes along with their respective promoters can be substituted by a foreign promoter and a transgene of interest up to 4.5 kb in size [41].

Production of rAAV vectors is a complex process because it not only requires that the AAV *rep* and *cap* genes are deleted and replaced, but also the necessary helper virus

activities have to be provided by the cell line. At the moment, such conditions are produced through transfection of a "helper plasmid" containing the well-characterized Ad helper genes (VA-RNA, E2A and E4) along with a "trans plasmid" containing the AAV *rep* and *cap* genes. A third "cis plasmid" containing the transgene between AAV2 ITRs is cotransfected with the other two plasmids into HEK 293T cells, which is a human cell line constitutively expressing the remaining Ad helper genes (E1A and E1B) that are required for vector production [36]. This technique is referred to as a "Triple Transfection" which results in amplification and packaging of rAAV vectors that are replication-deficient and harbour a foreign DNA promoter and transgene [1] (Figure 2.2). The triple transfection system abolishes the need for a helper virus to be present, thus eliminating the problem of rAAV vector preparation contamination with helper viruses [42].

All AAV serotypes can be produced as rAAV vectors using the methods described above. Only the *cap* gene of AAV on the trans plasmid needs to be replaced by an AAV serotype specific cap. It should be noted that the majority of rAAV vector ITRs as well as the *rep* ORF are typically derived from the well characterized AAV2. This method of combining the ITRs of AAV2 with the capsid of another AAV serotype to produce a different rAAV vector called pseudotyping or cross-packaging [43]. An example of this is the rAAV vector AAV2/5 which contains the ITRs from AAV2 and the *cap* gene from AAV5.

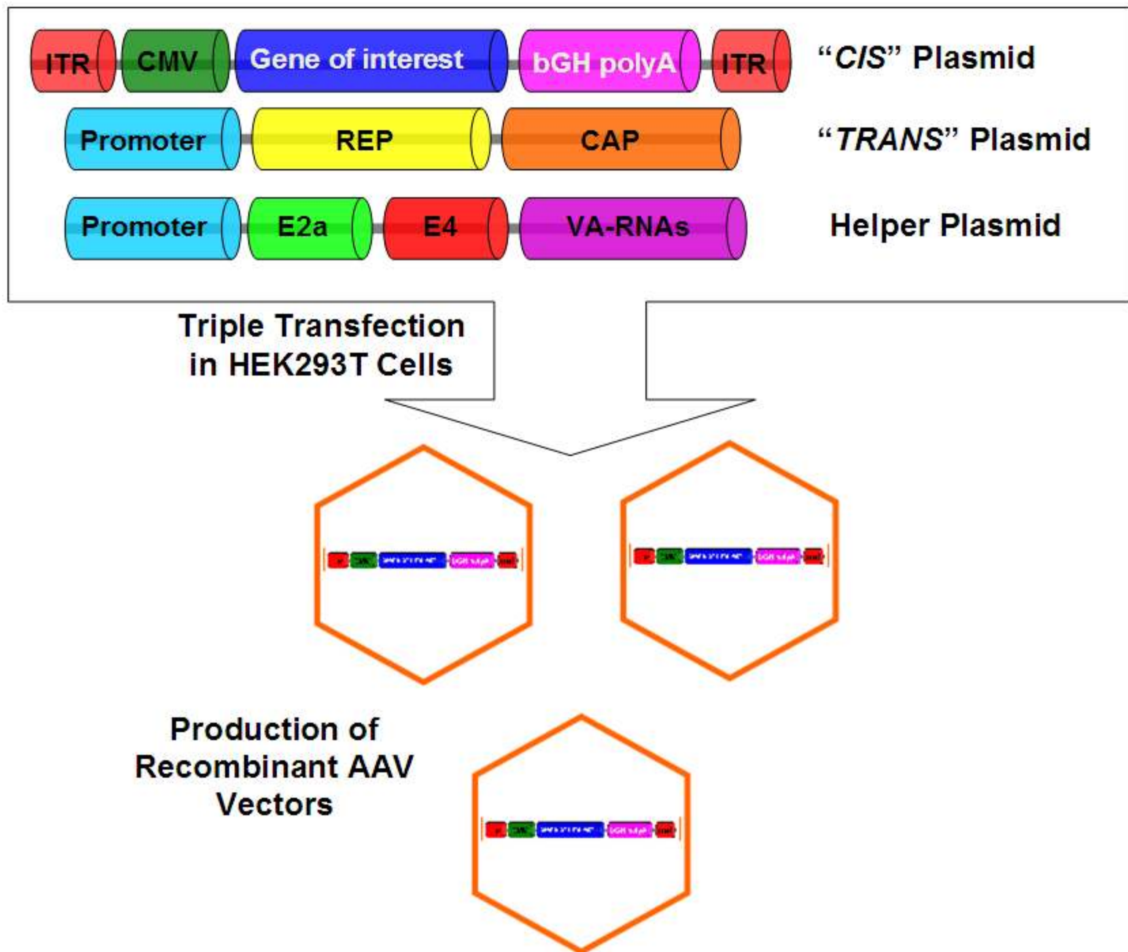


Figure 2.2 – Production of Recombinant AAV Vectors
Schematic representation of triple transfection system utilized for the production of rAAV vectors. System involves the use of a transgene expressing *cis* plasmid, a packaging *trans* plasmid and a helper plasmid containing Adenoviral genes need for replication. Co-transfection leads to the production of rAAV expressing a gene of interest along with the choice of capsid.

2.3 – AAV Vectors for Gene Therapy

The fundamental concept of gene therapy is to treat disease by delivering a nucleotide sequence into a target cell. This nucleotide sequence can then modify the cellular function directly or cause the cell to express a secreted protein in order to cure or improve the status of a disease. In general the two approaches employed for gene therapy are the use of viral vectors, or the use of non-viral vectors. Viral vector systems include AAV, Ad, Herpes simplex virus, Lentivirus and Retroviruses [37]. The non-viral vector systems use naked plasmid DNA or DNA that is coated by a lipid or polyethylene glycol (PEG) to prevent degradation of the DNA [44].

The design and development of all gene therapy vectors should incorporate elements of safety as well as efficiency and for these reasons AAV is a prime candidate. AAV has emerged as a preferred viral vector for gene therapy due to its capacity to transduce both dividing and non-dividing cells efficiently in addition to its low pathogenic risk and broad host range. Another key feature of AAV is that the vector genomes possess the ability to persist in cells in an unintegrated state, usually as an episome for the entire lifetime of the cell. This suggests that the best targets for AAV vectors are non-dividing cells, terminally differentiated cells or slow-growing cells. For these reasons AAV vectors are used in treating chronic diseases where gene expression will be required for a prolonged period of time and infrequent delivery is a necessity. Support for these findings is reflected in current clinical trial applications of AAV vectors for the treatment of inherited and acquired genetic diseases (Table 2.3) [45]. Optimal targets for AAV

vectors include cells and tissues like muscle, liver, brain, retinal pigment and photoreceptors of the eye (e.g. Leber´s congenital amaurosis), lung and airway epithelial cells (e.g. Cystic Fribrosis). In some cases, a convenient location to express a secreted protein is used as a target, as with the treatment of haemophilia B by secretion of factor IX from either the liver or muscle.

Table 2.3 – Overview of rAAV Vectors Used in Clinical Trials

Disease	Transgene Product	Serotype	Route of Administration	Clinical Trial
AAV Clinical Trials for Inherited Diseases				
α1 antitrypsin deficiency	α1 antitrypsin	AAV2 AAV1	Intramuscular	Phase I/II
Batten's disease	CLN2	AAV2 AAVrh10	Direct intracranial administration	Phase I
Canavan's disease	Aspartoacylase	AAV2	Direct intracranial administration	Phase I
Cystic fibrosis	CFTR	AAV2	Direct instillation to maxillary sinus, bronchoscopy to right lower lobe, aerosol to whole lung	Phase I/II
Haemophilia B	Factor IX	AAV2	Intramuscular Hepatic	Phase I/II
		AAV8	Intravenous	
Leber's congenital amaurosis	RPE65	AAV2	Subretinal	Phase I/II
LPL deficiency	LPL	AAV1	Intramuscular	Phase I/II
Pompe's disease	GAA	AAV1	Intra-diaphragmatic injections	Phase I/II
Muscular dystrophy: Duchenne	Microdystrophin	AAV1–AAV2 hybrid	Intramuscular	Phase I
Muscular dystrophy: limb girdle	α-sarcoglycan	AAV1	Two to six separate injections into the selected muscle	Phase I
AAV Clinical Trials for Acquired Diseases				
Severe heart failure	SERCA2a	AAV1 AAV6	Antegrade epicardial coronary artery infusion	Phase I/II
Parkinson's disease	AADC GAD Neutrophin	AAV2	Intracranial	Phase I/II
Age-related macular degeneration	sFLT01	AAV2	Intravitreal injection	Phase I
Rheumatoid arthritis	TNFR-Fc	AAV2	Intra-articular	Phase I

<u>Abbreviations:</u> AADC, aromatic-L-amino-acid decarboxylase; CFTR, cystic fibrosis transmembrane regulator; CLN2, also known as tripeptidyl peptidase 1 (TPP1); GAA, acid α-glucosidase; GAD, glutamic acid decarboxylase; LPL, lipoprotein lipase; RPE65, retinal pigment epithelium-specific protein 65 kDA; SERCA2a, sarcoplasmic reticulum calcium ATPase 2a; sFLT01, portion of the vascular endothelial growth factor natural receptor; TNFR-Fc, tumour necrosis factor receptor-immunoglobulin Fc fragment fusion protein. (Modified from Mingozzi, F. and K.A. High 2011 [45])

2.4 – AAV Vectors and Immune Evasion

Initially, interest in AAV vectors emerged based on their use for gene therapy applications due to their nonpathogenic nature and long term gene expression, in which the majority of this work was done with vectors derived from AAV2. During early *in vivo* experiments, stable transgene expression and poor immune responses to β-galactosidase from *Escherichia coli* was observed in mice following IM injections of AAV2-LacZ [46]. This lack of an immune response by the host to AAV vector encoded antigens was also observed with a variety of other serotypes and transgene products carried out in mice and larger animals [47]. As a result, a number of methods have been suggested to explain the apparent lack of T-cell responses and immune evasion following *in vivo* gene transfer with AAV vectors. Methods to account for this include T-cell ignorance, anergy, deletion or suppression.

Preliminary results have shown that AAV vectors induce immunological ignorance based on inadequate innate immune activation and poor transduction of antigen presenting cells (APCs), thereby leading to a lack of T cell priming towards the encoded transgene [48]. For successful $CD8^+$ T cell priming there are three basic signals that are essential: antigen presented on major histocompatibility complex (MHC), costimulatory molecules CD80/86 and CD40 and appropriate cytokine signals. In some cases there can be antigen presentation devoid of adequate upregulation of costimulatory factors which results in anergy or deletion of T cells. This was demonstrated in an AAV vaccine study where adoptive transfer of APCs into CD40L deficient mice the resulted in presence of

antigen-specific T cells that were non-functional, meaning that they are unable to secrete cytokines (principally IL-2) or proliferate in response to antigenic stimulation [49]. Another study involving AAV showed that improper costimulation by using an anti-CD40L monoclonal antibody to block the APC-T cell interaction in mice was enough to bypass immune responses to AAV vectors [50]. More recent studies provide evidence that AAV vectors can generate functionally aberrant T cell populations which fail to proliferate and expand upon secondary exposure to antigen [47, 51].

Accompanying ignorance, anergy and deletion there is an alternative method of immune tolerance that is based on active suppression. In suppression a special class of T-cells called "regulatory T-cells" (Tregs) are able to suppress activated effector $CD8^+$ T-cells allowing for the induction immunological tolerance. The dominant subclass of Tregs is characterized as having high expression CD25 and the transcription factor Foxp3, therefore they are termed $CD4^+CD25^+Foxp3^+$ [45]. Conclusive evidence has been shown that tolerance induction by Tregs to a specific transgene can be achieved with AAV vector serotypes AAV2/2 and AAV2/8. This was found in both mice and NHPs through hepatic gene transfer where $CD4^+CD25^+Foxp3^+$ was found to be responsible for suppression of antigen-specific T cell responses and antibody formation [1, 18]. It was also shown in mice using AAV2/8 that Tregs in the liver secrete the immunosuppressive cytokine IL-10 in response to antigen, which in turn suppresses the CD8+ cytotoxic T-lymphocyte (CTL) response [52]. Taken together these findings describe a variety of methods as to how AAV can evade the host immune response.

2.5 – AAV as a Vaccine Vector

Due to the apparent lack of T cell responses and immune evasion following *in vivo* gene transfer with AAV vectors, *in vivo* applications for AAV vectors in gene therapy expanded and it was realized that the immunological tolerance of AAV transgene products was misleading and not absolute. Many instances were identified in which vibrant T cell responses to AAV encoded transgene products were amplified by the host [50, 53, 54]. It was established that there are certain features responsible for affecting the immunological response to the expressed transgene. One group showed that the strength of the T cell response after AAV gene transfer was dependent on subcellular localization of the protein being expressed [55]. The route of administration and dose of the AAV vector was also found to contribute to both the strength and magnitude of T and B cell responses to the transgene [54, 56]. Another group determined that inflammation at the site of AAV delivery promotes antigen-specific immune responses to the transgene [57]. The finding that B cell responses are much stronger and more frequently produced than $CD8^+$ T cell responses to AAV encoded transgenes has remained a constant observation as well [34, 58, 59]. These findings have caused researchers to explore AAV vectors as vaccine platforms to an assortment of both non-infectious and infectious diseases, derived from the evidence that they can be developed to stimulate a transgenic immune responses [5, 60, 61].

2.6 – Risks Associated with the Use of AAV Vectors

Although AAV vectors have had a solid success rate in small and large animal models, a variety of problems has limited the extent of successful applications in humans. Over the past decade, clinical studies have identified several impediments not only to gene therapy but vaccine applications as well. A combination of either the vector, transgene or target tissue can cause the majority of clinical problems. Some examples of possible risks associated with AAV based therapies include gene silencing, insertional mutagenesis, phenotoxicity, immunotoxicity and horizontal or vertical transmission of donor DNA [45].

3.0 – Influenza A Viruses

3.1 – Overview and General Properties of Influenza A Viruses

Influenza A viruses belong to the family *Orthomyxoviridae* and contain an enveloped, negative sense single-stranded RNA genome with 8 segments encoding 10 genes: hemagglutinin (HA), neuraminidase (NA), matrix proteins (M1 and M2), polymerase proteins (PB1, PB2, PA), non-structural proteins (NS1 and NS2), and the nucleoprotein (NP) [62-64]. The viral envelope includes the HA, NA, and M2 proteins. The HA glycoprotein mediates attachment, fusion and entry of the virus by binding to sialic acid receptors on the surface of epithelial cells [65]. The NA glycoprotein cleaves sialic acid residues on the surface host and viral membranes allowing mature virions to be released from infected cells [66]. The matrix proteins M1 and M2 are essential for the structural integrity of influenza virions [67].

Currently, there are 16 HA subtypes (H1-16) and nine NA subtypes (N1-9) emanating in wild bird populations, and strains from two virus subtypes (H1N1 and H3N2) are found to circulate in humans [68]. The accepted nomenclature for influenza viruses indicates the combination of external glycoproteins (HA and NA) to identify virus subtypes, despite the origin of the internal genes. Furthermore, each virus strain is classified with the location and year of isolation for further reference (e.g. A/Hanoi/30408/2005 (H5N1)). The HA subtypes are divided into two groups based on their antigenic features and structural characteristics [69, 70]. Group 1 contains the H1a, H1b, H5 and H9 HA subtypes whereas Group 2 is comprised of the H3 and H7 HA subtypes. The antigenic

progression of seasonal influenza A viruses in humans arises from "antigenic drift", where antibody-mediated immune selection pressure results in the accumulation of mutations and is portrayed by the seasonal selection of new strains containing amino acid changes in the HA and NA. These new strains are responsible for seasonal influenza epidemics because the changes in HA and NA can adequately overcome pre-existing immunity in humans. "Antigenic shift", caused by the reassortment of viral genomes resulting in the generation of a new subtype, causes more extreme changes in the HA subtype and has been associated with the development of pandemic viruses [71].

3.2 – Avian Influenza Viruses

The majority of influenza A viruses are found in wild bird reservoirs and only a few subtypes are pathogenic in humans. Low pathogenic avian influenza (LPAI) A viruses can reside in the respiratory and gastrointestinal systems of aquatic birds as asymptomatic or mild infections and are periodically transmitted to domestic poultry or urban bird populations. Highly pathogenic avian influenza (HPAI) A viruses were first recognized in 1997, in chickens from Hong Kong where several clinical cases in humans were identified in individuals who lived or worked in close proximity to the infected birds and six human fatalities were reported [72]. While human-to-human transmission was not observed or reported, domestic poultry flocks were culled as precaution. This was done to avoid a potential influenza virus pandemic and also to stop cross-transmission of the HPAI H5N1 virus to humans. In 2003, a novel HPAI H5N1 strain appeared in domestic and wild bird populations in South East Asia. More than 500 human cases have been

reported in Asia, the Middle East, and Africa and isolated cases of human-to-human transmission have been confirmed in Indonesia, Pakistan, and China [73]. Although the transmission of H5N1 viruses among humans is inadequate, the high mortality rate (>60%) suggests that these viruses could be extremely dangerous should they become more efficient at transmission in humans [74].

3.3 – Prevention of an Avian Influenza Pandemic

Due to the risk of a highly virulent avian influenza virus, the expansion of current vaccines and the advancement of alternative preventative and therapeutic strategies are not only important but also necessary to prepare for the emergence of potential pandemic avian influenza viruses like H5N1 or other HPAI viruses such as H3N7, H7N7, and H9N2. A new approach for protection against influenza virus is to use AAV vectors as vaccine models. AAV has been recently employed as a vaccine model against other strains of influenza with promising results *in vivo* [60]. AAV has also been shown to induce strong B-cell responses which are a correlate of protection against influenza viruses giving rise to their potential application in humans [75].

4.0 – Overview of Objectives and Statement of Hypothesis

Vectors derived from AAV can deliver genes to a variety of tissues *in vivo* and recent findings have also promoted the evaluation of AAV as potential vaccine platform, due to its long-term expression profile which can stimulate robust immune responses. Currently, clinical trials are taking place using AAV as a vector for the treatment of ailments such as Parkinson´s disease, Cystic fibrosis, Leber´s congenital amaurosis, HIV infection, and various other genetic disorders [30]. The majority of these rAAV vectors are based on the well characterized human AAV2 which is seroprevalent in up to 80% of the human population with AAV2 neutralizing antibodies found in 35% of them [41]. Therefore, it is of great importance to find novel AAV serotypes to which the majority of humans do not possess neutralizing antibodies that can offer better alternatives for gene therapy and vaccine development.

The AAV capsid proteins encoded by the *cap* gene are shown to be the primary determinant for tissue tropism and constitute an important target for the immune response. Certain AAV serotypes are found to be less immunogenic than others, making them better candidates for gene therapy and less efficient as vaccine vectors or vice versa. In the past, AAV was mainly isolated from contaminated adenoviral stocks or from young, sick animals or children [45, 52, 76, 77]. More recently, isolation of new AAV isolates was extensively performed using PCR amplification of AAV sequences from genomic DNA of different animal species [8, 26, 29, 78].

Our lab has isolated novel AAV sequences from porcine tissues identified by PCR using genome walking strategies and then produced vectors based on these novel AAV sequences, characterizing them *in vitro* and *in vivo*.

It was hypothesized that: Novel porcine AAV serotypes will be efficient for either vaccine or gene therapy applications based on their level of immunogenicity.

The objectives of the studies in this thesis were to:

(1) Characterize tissue tropisms and transduction efficiency of the novel porcine AAVs *in vitro* as well as *in vivo*

(2) Evaluate immune responses to novel AAVs *in vivo*

(3) Identify "immunogenic" AAVs for vaccine studies and "non-immunogenic" AAVs for gene therapy.

This was done by constructing novel porcine AAV vectors in addition to existing AAV vectors that expressed either the LacZ transgene or the hemagglutinin (HA) from the highly pathogenic avian influenza H5N1 strain A/Hanoi/30408/2005 and carrying out vaccinations as well as challenge studies in mice. Mice vaccinated with LacZ expressing vectors were used for tropism studies and mice vaccinated with HA were used immunological studies.

5.0 – Materials and Methods

5.1 – Isolation of Genomic DNA from Porcine Tissues and Amplification of AAV Sequences

Different porcine tissues including the heart, lung, spleen, gut, and liver were screened for the presence of AAV sequences and this was done in collaboration with fellow labmate Alex Bello. Porcine tissue samples were obtained by Chris Kranendonk at the Public Health Agency of Canada (PHAC) from different areas such as private rural farms and industrial slaughterhouses where samples were provided. Approximately 25 mg of each tissue was used to obtain genomic DNA and was extracted using QIAamp® DNA Mini Kit (QIAGEN, Valencia, CA, USA).

Primers SIG+ (5´-GGTAATTCCTCGGGAAATTGGCATT-3´) and SIG- (5´-GAATCCCCAGTTGTTGTTGATGAGTC-3´) were used to PCR amplify short fragments of AAV capsid DNA referred as the "signature region". This region is highly conserved but the core sequences fluctuate and are unique to each known AAV serotype [77]. The resulting short DNA fragments ranging from 250-270bp corresponded to novel porcine AAV sequences generating AAVpo1, -po4, -po5 and -po6 fragments isolated in collaboration with Alex Bello (Figure 5.1). BLAST analysis was performed on the porcine AAV sequences displaying significant differences from previously described AAV sequences available on GenBank. The 5´ sequence of the AAV *cap* was isolated using the primers RC+ (5´-GGTGCGTAAACTGGACCAATGAGAAC-3´) [26] and SIG- resulting in a 1.64 kb fragment that contained the end of the AAV *rep* gene and

beginning of AAV *cap* region. Three consecutive primers were then produced based on conserved regions of the AAV genome in addition to a reverse degenerate primer in order to isolate the 3´ end of the *cap* gene. The process was carried out in a three step nested Thermal Asymmetric Interlaced (TAIL) PCR [79] using HotStarTaq DNA Polymerase (QIAGEN, Valencia, CA, USA) and PfuUltra™ High-fidelity DNA Polymerase (Agilent Technologies, Cedar Creek, TX, USA) (Table 5.1). The sequences of the three consecutive primers were: PAAVSP1+ (5´-GGARATTGGCATTGCGATTCC-3´), PAAVSP2+ (5´-GACTTCAACCGCTTCCACAGCCAC-3´), and PAAVSP3+ (5´-GACTCATCAACAACCWACTGGGG-3´). The sequence of the degenerate primer CED- was: 5´-ACTGAMACGAAT(H/-)AMMCGGTTTATTGA-3´. Using these primers a 1.4 kb fragment was obtained that overlapped with the 1.64 kb 3´ *rep* end sequence. To obtain the full length *cap* gene from porcine tissues in one PCR step, specific primers were also designed with the following sequences: PO1CAPBEGIN+ (5´-ATGTAGTGGATCTTGACGATG-3´) and PO1CAPEND- (5´-CATGTGACAGGATAGGGTTA-3´). Please note that this same process was used to obtain other porcine AAV serotypes (AAV-po4, -po5, and -po6).

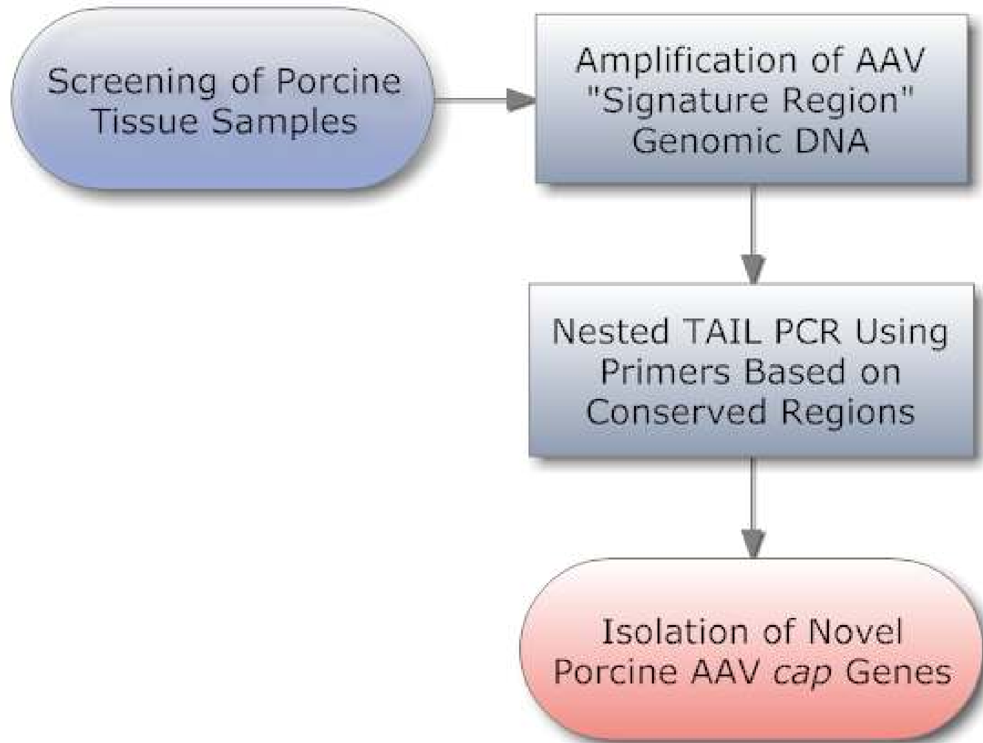


Figure 5.1 – Overview of Isolation of Novel Porcine AAVs
Flow diagram outlining procedures involved with isolating novel porcine AAV cap genes from porcine tissues.

Table 5.1 – Nested TAIL PCR Protocol for Isolation of AAV *cap* Gene

Reaction 1	Amount (µL)	Step #	Temperature (°C)	Time	Cycles
1.5 uM Specific Primer	3	1	94	15 min	
2.0 uM Reverse Degenerate Primer	4	2	94	45 sec	
Buffer (10x)	2	3	Primer Tm -5	30 sec	
100uM dNTPs	1	4	72	1 min / kb	Go to Step 2 (4X)
HotStarTaq (diluted 5 fold)	2	5	94	45 sec	
MgCl2	0.5	6	30	30 sec	
H_20	5.5	7	72	1 min / kb	
PfuUltra (diluted 100 fold)	1	8	94	45 sec	
Porcine DNA	1	9	44	30 sec	
Total Volume	20	10	72	1 min / kb	Go to Step 8 (9X)
		11	94	45 sec	
		12	Primer Tm -5	30 sec	
		13	72	1 min / kb	Go to Step 11 (1X)
		14	94	45 sec	
		15	44	30 sec	
		16	72	1 min / kb	Go to Step 11 (11X)
		17	72	10 min	
		18	4	Hold	

Table 5.1 – Nested TAIL PCR Protocol for Isolation of AAV *cap* Gene (Continued)

Reaction 2	Amount (µL)	Step #	Temperature (°C)	Time	Cycles
1.5 uM Specific Primer	3.75	1	95	15 min	
2.0 uM Reverse Degenerate Primer	5	2	94	45 sec	
Buffer (10x)	2.5	3	Primer Tm -5	30 sec	
100uM dNTPs	1	4	72	1 min / kb	Go to Step 2 (1X)
HotStarTaq (diluted 5 fold)	1	5	94	45 sec	
MgCl2	0.5	6	44	30 sec	
$H_2$0	9.25	7	72	1 min / kb	Go to Step 2 (10X)
PfuUltra (diluted 100 fold)	1	8	72	10 min	
DNA (from Reaction 1)	1	9	4	Hold	
Total Volume	25				

Reaction 3	Amount (µL)	Step #	Temperature (°C)	Time	Cycles
1.5 uM Specific Primer	1	1	95	10 min	
2.0 uM Reverse Degenerate Primer	1	2	94	1 min	
Buffer (10x)	5	3	Primer Tm -5	1 min	
100uM dNTPs	1	4	72	1 min / kb	Go to Step 2 (1X)
HotStarTaq (diluted 5 fold)	1.5	5	94	1 min	
MgCl2	0.5	6	44	1 min	
$H_2$0	20	7	72	1 min / kb	Go to Step 2 (10X)
PfuUltra (diluted 100 fold)	1	8	72	10 min	
DNA (from Reaction 2)	1	9	4	Hold	
Total Volume	32				

5.2 – Cloning Procedures for PCR Products Involved with AAV Vector Production

All PCR amplified fragments obtained from the steps above were loaded onto a 1% agarose gel and run at 100 V for approximately 1 hour in TAE buffer (Life Technologies, Carlsbad, CA, USA). 2 Log Ladder (Life Technologies) was used as a DNA marker. After 1 hour, the agarose gel was imaged with the corresponding bands being excised and gel purified using QIAguick® Gel Extraction Kit (QIAGEN) in accordance with the manufacturer's recommended protocol. The purified PCR fragments were later cloned into pCR®2.1-TOPO® Vector (Invitrogen, Carlsbad, CA, USA). The gel extracted PCR product was incubated with TAQ DNA polymerase (Life Technologies) along with dATPs for 30 minutes at 72˚C.

Cloning of the PCR products was carried out using the Topo TA® Cloning Kit and the pCR®2.1-TOPO® Vector (Invitrogen) as described. The resulting reaction mixture was then transformed into One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen) using the manufacturer's recommended protocol. Lysogeny Broth (LB) agar plates supplemented with 100µg/ml of ampicillin, in addition to X-Gal for screening using blue-white selection, were inoculated with the transformed cells and incubated at 37˚C overnight. The next day, white colonies were selected to inoculate 5ml of LB Lenox (1.5% NaCl) Broth and placed in a 37˚C shaking incubator set to 250 rpm overnight.

All plasmid DNA purification steps were performed using QIAprep® Miniprep Kits (Qiagen) in accordance with the manufacturer's recommended protocol. Samples were

eluted with 50 µL of Buffer EB (Qiagen) and restriction digests were performed using enzymes purchased from New England Biolabs (NEB, Pickering, Ontario) according to manufacturer's recommended protocols.

The porcine AAV *cap* genes (po1, po4, po5 and po6) were screened with *BamH*I and *Swa*I (NEB) and suitable clones were sent for sequencing performed by the DNA Core Facility at the National Microbiology Laboratory (NML) (Winnipeg, Manitoba, Canada) using primers ordered from IDT DNA Technologies. All DNA samples were diluted to a final concentration 150 uM and all primers were diluted to 1 µM in deionized water. Following conformation by sequencing, the isolated porcine AAV *cap* genes were digested with *Swa*I and *Not*I, and the resulting fragments were ligated into the pACK2 packaging plasmid (explained below).

Ligations of the porcine AAV *cap* genes (po1, po4, po5, po6) into pACK2 were performed by the use of low melt agarose "in-gel" ligations. The procedure involved separation of the digested constructs on a 0.7% low melt agarose gel (Life Technologies), then bands of interest were excised and frozen at -80°C for 5 minutes after which they bands were melted at 72°C. Four ligation reactions were used, each with a different ratio of vector to insert (7:0, 3:7, 2:8, and 1:9). T4 DNA ligase (NEB) was prepared on ice according to manufacturer's recommended protocol and was added to the melted bands at 37°C. After vigorous mixing, the ligation reactions were left overnight at 16°C and the following day the ligations were heated to 37°C and transformations were carried out

with competent cells (Invitrogen). Competent cells were inoculated onto LB agar plates supplemented with 100µg/ml of ampicillin and incubated at 37˚C overnight. The next day, colonies were selected to inoculate 5ml of LB Lenox (1.5% NaCl) Broth and placed in a 37˚C shaking incubator set to 250 rpm overnight. Plasmid DNA purification steps were performed using QIAprep® Miniprep Kits (Qiagen) as previously explained.

5.3 – Hybrid Porcine AAV Particle Production and Purification

5.3.1 – Plasmids Involved with Production of Hybrid Porcine AAV Particles

Hybrid AAV particles were generated using two expression plasmids, one of which was the *cis*-plasmid that expressed the desired transgene along with the AAV2 *rep* gene and the other *trans*-plasmid expressed the porcine AAV *cap* genes (e.g. AAVpo1/po4/po5/po6). AAV cap genes from two known serotypes AAV8 and AAVrh.32.33 (University of Pennsylvania) from rhesus monkeys were used to produce control vectors for immunological characterization studies. The porcine AAV *cap* genes were cloned into the p600 *trans* packaging plasmid in place of the AAV2 *cap* gene using the *Swa*I and *Not*I restriction sites (described above), producing the pACK2/po1, 4, 5 and 6 plasmids.

An AAV2-CMV-LacZ plasmid [36], expressing the *lacZ* reporter gene from a CMV promoter and flanked by AAV2 ITRs was employed as the *cis* plasmid containing the recombinant AAV genome. The AAV2-CMV-LacZ plasmid was used for characterization studies involving tissue transduction and tropism of novel porcine

AAVs.

The HA gene from Hanoi 2005 strain of Influenza virus was cloned into another expression plasmid, AAV2-CMV-H5N1-HA. This plasmid also employed a CMV promoter to express the HA gene and was flanked by AAV2 ITRs. The AAV2-CMV-H5N1-HA plasmid was used for studies involving immunological characterization of novel porcine AAVs.

Recombinant AAV genomes containing AAV2 ITRs expressing either the *lacZ* reporter gene or HA gene via a CMV promoter were packaged by triple transfection of human embryonic kidney (HEK) 293T cells.

5.3.2 – Cell Culture and DNA Transfections

All cell culture work was carried out in a Biological Safety Cabinet (BSC) under aseptic conditions and techniques. HEK 293T cells were sustained in Dulbecco´s Modified Eagle Medium (DMEM) (Sigma-Aldrich, Oakville, Ontario) supplemented with 10% Fetal Bovine Serum (FBS, Wisent, St. Bruno, Quebec), 1% penicillin/streptomycin (Gibco, Carlsbad, California), 1% L-glutamate (Gibco), and 1% NaPyr (Gibco). Madin-Darby canine kidney cells (MDCK) were sustained in Eagle's Minimum Essential Medium (MEM) (Sigma-Aldrich) supplemented with 10% FBS (Wisent) and 1% penicillin/streptomycin (Gibco). All cell lines (ATCC, Manassas, VA, USA) were cultured at 37°C in humid atmosphere containing 5% CO_2. Cells were passaged when confluent by removing culture media and adding an appropriate volume of either 0.05%

or 0.25% Trypsin-EDTA (1x) (Invitrogen) to detach cells.

DNA triple transfections were carried out using either the pAAV2.1-CMV-LacZ or pAAV2.1-CMV-H5N1-HA "*cis* plasmid", the pACK2/po1 (-po4, -po5 and -po6) or pACK2/5 [42] packaging "*trans* plasmid" and the pAd-DeltaF6 "helper plasmid" that contains adenoviral genes (E2a, E4, VA-RNA) essential to drive AAV replication. Calcium Phosphate (CalPhos™) Mammalian Transfection Kits from Clonetech (Mountain View, California, USA) were used for DNA transfections of HEK 293T cells and they were performed in 150mm x 25mm tissue culture dishes (Corning, NY, USA) as described previously [36, 37] according to manufacturer's recommended protocols.

40 plates of HEK 293T cells were seeded at a concentration of $6.0x10^6$ cells/plate 24hrs prior to transfection. The next day the cells were between 60-80% confluent and 2 hours prior to transfection the cell culture media was changed and replaced with fresh media. Triple transfections were performed with the three (*cis, trans* and helper) packaging plasmids using CalPhos™ transfection kits following manufacturer's guidelines described below:

(Concentrations reflect amounts based on a single 150mm x 25mm tissue culture dish)

Solution A: components are added in the following order
120µg Plasmid DNA (30µg *cis,* 30µg *trans* and 60µg helper)
1.2ml Sterile H_20
0.2ml 2M Calcium Solution

Solution B:
1.6ml 2X HEPES-Buffered Saline (HBS)

After preparation, Solution A is slowly vortexed while Solution B is added dropwise. Once both solutions are mixed together they are left for 20 min at room temperature to incubate. Following the 20 min incubation the solution (now slightly cloudy) is vortexed and added dropwise to each plate. The plates are moved from side to side and front to back to ensure proper distribution of the transfection solution. The plates are then are incubated overnight for 16 hours at 37°C in humid atmosphere containing 5% CO_2. The next morning the media containing the transfection solution is removed and replaced with fresh media and the plates are incubated for an additional 48 hours at 37°C in humid atmosphere containing 5% CO_2. 48 hours later, the transfected cells were harvested in 500ml screw cap conical bottom centrifuge tubes (Corning) and pelleted by centrifugation at a speed of 500 g for 10min after which the supernatants were removed and the tubes were stored at -80°C.

5.3.3 – Purification of AAV Particles

Frozen AAV preparations (preps) stored at -80°C, were removed and thawed in a 37°C water bath. Once thawed AAV preps were resuspended in lysis buffer (50 mM Tris/1 mM $MgCl_2$ (pH 7.5)) and brought up to 25ml. The tubes were transferred to 50ml conical centrifuge tubes (BD Falcon™, Mississauga, ON) where cellular and nuclear membranes were destroyed by 4 consecutive freeze and thaw cycles. The suspensions were then treated with 5000 Units of DNaseI (Invitrogen) for 30 minutes at 37°C to eliminate genomic and plasmid DNA contaminants in the vector preparations. Following DNaseI treatment, 1.25ml of 10% deoxycolate (DOC) (used as detergent and isolating agent for membrane proteins) was added to the suspension and is incubated for 10

minutes at 37°C then placed on ice.

5.3.4 – Cesium Chloride Gradient Purification of AAV Particles

0.454g/ml of CsCl powder (Fisher Scientific, Toronto, ON) was added to the AAV cell lysate suspensions now on ice. CsCl gradient centrifugation was used to remove cellular debris and to concentrate AAV particles. Two solutions of CsCl (Fisher Scientific) were prepared for the gradient, a 1.4 density (548.3mg/ml) CsCl and a 1.6 density (816.5mg/ml) CsCl both of which were filter sterilized using a pore size of 0.45µm (Corning). Gradients were setup using open-top 89 x 25 mm thinwall polyallomer tubes (Beckman Coulter, Mississauga, ON) where 9ml of 1.4 CsCl was added first to the tubes and 8.5ml of the 1.6 CsCl was then slowly added underneath the 1.4 CsCl to create a gradient. The AAV cell lysate suspensions were carefully added on top of the CsCl gradients and the tubes were loaded into a Swinging Bucket SW 32 Ti Rotor (Beckman). The tubes were centrifuged using an Optima L-90K Ultracentrifuge (Beckman) for 18 hours at a temperature of 15°C and a speed of 26,000 rpm. Once the spin was completed, the polyallomer tubes were removed from the rotor and placed onto holding stands in the BSC.

5.3.5 – Fractionation of Cesium Chloride Gradients

The exterior of each polyallomer tube was cleaned using WEBCOL™ Alcohol Prep Pads (Covidien, Mansfield, MA USA) prior to fractionation. An 18G x 1½ inch needle (BD, Franklin Lakes, NJ USA) was used for the fractionation steps and was inserted into the bottom of each polyallomer tube. Fractions were collected upon needle insertion with the

first 5ml of suspension being collected in a 15ml conical centrifuge tubes (BD Falcon™) and the remaining amount was collected in 1.7ml fractions in 2.0ml graduated flat top microtubes (Diamed, Mississauga, ON). Following collection, the refractive index of each fraction was measured using a Tabletop 3L Refractometer (Fisher). Fractions within the refractive index range of 1.3750-1.3680 in which the majority of AAV serotypes are found based on their buoyant density in CsCl [17], were kept and pooled together for further processing.

8.9ml of the pooled fractions were transferred to new 16 x 60 mm polyallomer OptiSeal™ tubes (Beckman) and were loaded into a Fixed Angle Type 90 Ti Rotor (Beckman). The tubes were centrifuged in an Optima L-90K Ultracentrifuge for 18 hours at a temperature of 15°C and a speed of 62,000 rpm. Following the spin, the same procedures were followed as described above only this time 0.5ml fractions were collected in 1.5ml graduated flat top microtubes (Diamed) to be read by the refractometer. Fractions within range were pooled together then transferred to Amicon Ultra-15 Centrifugal Filter Units (Millipore, Billerica, MA USA) where the solutions were washed and concentrated according to the manufacture's recommended protocols. After being washed and concentrated the solutions were aliquoted in the appropriate volume into 2.0ml screw cap microtubes (SARSTEDT, Montreal, QC) and stored at -80°C until use.

5.3.6 – Titering of AAV Particles by Real Time PCR

The produced hybrid AAV particles are replication deficient and lack the *rep* and *cap*

genes necessary to replicate and produce progeny virus. Therefore, to determine the viral titre of the AAV preps the number of genome copies (GC) is determined by Real Time PCR (RT-PCR) using a known concentration standard based on the AAV expression plasmid.

To prepare the AAV preps for RT-PCR, 10µl of the AAV prep diluted 100 fold was added to 89µl of H_2O and 1µl (40 units) of DNaseI for 1 hour at 37°C in a waterbath. Following the 1 hour incubation the reactions were placed on a heating block set at 99°C for 5 minutes and the transferred to an ice bucket for 10 minutes.

The concentration standards were prepared from the respective transgene encoding plasmids by carrying out restriction digests for 1 hour at 37°C in a waterbath with *Not*I to linearize the plasmids. The linearized plasmids were then purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacture's protocols. The DNA concentration of the linearized plasmids was then determined by a NanoDrop 1000 micro-volume spectrophotometer (Fisher). The plasmid concentrations were converted based on their formula weight and molar concentration (M) to determine their copy number/µl. Samples were diluted accordingly to a starting dilution of $5x10^6$ copies/µl from which the concentration standard was made.

RT-PCR was carried out on the prepared AAV DNA samples along with the concentration standards. Two primers (BGH FWD and BGH REV) along with a dual

labelled probe (DLP) (Eurofins MWG Operon, Huntsville, AL, USA) specific to the bovine growth hormone (BGH) polyadenylation (polyA) signal was used for the RT-PCR. The BGH polyA signal was present in the transgene encoding plasmids and therefore the hybrid AAV genomic DNA as well. Quantitect® Probe RT-PCR Master Mix (QIAGEN) was used in conjunction with primers and the DLP to make up the RT-PCR reaction master mix. 15µl aliquots of the prepared master mix along with 10µl of sample DNA was added to a white 96 well optical plate (Roche, Laval, QC) and loaded into LightCycler® 480 Real-Time PCR System (Roche) as described below in Table 5.2. Upon completion of the RT-PCR run, the results were transferred to Microsoft Excel where the AAV titre was calculated based on a standard concentration curve and converted into genome copies (GC)/mL.

Table 5.3 – RT-PCR Reaction Mixture for Titering of Hybrid AAV

REAGENT	Amount (µL)	Step #	Temperature (°C)	Time	Cycles
Quantitect® Probe RT-PCR Master Mix	12.5	1	50	2 min	
BGH FWD Primer (100µM)	0.27	2	95	10 min	
BGH REV Primer (100µM)	0.27	3	95	15 sec	
Probe (2µM)	2.5	4	60	1 min	Go to Step 3 (40X)
DNA from Prepared Sample	10				
Total Reaction Volume	**25**				

BGH FWD: 5´-TCTAGTTGCCAGCCATCTGTTGT-3´
BGH REV: 5´-TGGGAGTGGCACCTTCCA-3´
Probe: (Dye, 6-FAM) 5´-TCCCCCGTGCCTTCCTTGACC-3´ (Quencher, TAMRA)

5.4 – Measurement of Protein Expression by Hybrid AAV Vectors

To confirm viral expression of the transgene, protein expression of each AAV vector was evaluated by western blotting. HEK 293T cells were cultured to 60-80% confluence in 6 well flat-bottom tissue culture plates (Corning) and were infected with recombinant AAV at a multiplicity of infection (M.O.I) of 60,000 (based on GC/mL as determined by RT-PCR). 72 hours post-infection, cell supernatants were discarded from each well and 200 µL of radioimmunoprecipitation (RIPA) buffer was added to each well to protect protein

from being degraded. Cell lysates were collected and analyzed under reducing conditions with 5x SDS gel loading buffer. Protein samples were separated on a 10% SDS-PAGE gel and wet transferred electroblotting using a Mini-PROTEAN Tetra Cell (Bio-Rad, Hercules, CA, USA) to a polyvinylidene fluoride (PVDF) membrane (GE Healthcare, Baie d'Urfe, QC) for 1 hour at 350mA.

The protein blots were incubated with the appropriate mouse immune sera as the primary antibody and were probed using a goat anti-mouse IgG horseradish peroxidase (HRP) conjugated secondary antibody (Fitzgerald Industries, North Acton, MA, USA). The expected protein bands were visualized via Amersham ECL Plus™ Western Blotting Detection Reagents and developed using Amersham Hyperfilm ECL (GE Healthcare).

5.5 – Electron Microscopy of Produced Hybrid AAV Particles

Novel porcine AAV samples along with an AAV2/5 control were purified by CsCl gradients. The samples were then prepared for electron microscopy by adsorption to glow discharged carbon coated formvar films on 400 mesh copper grids for one minute, and negatively contrasted with 2% methylamine tungstate (Nanoprobes, Yaphank, New York). Specimens were imaged in a FEI Tecnai 20 transmission electron microscope operating at 200 kV, at a nominal instrument magnification of 100000x (Daniel Beniac).

5.6 – Transduction Efficiency of Novel AAV Vectors *In Vitro*

To measure transduction efficiency and cell tropisms of the porcine vectors, various cell lines were infected with AAV vectors expressing LacZ. Cell lines were seeded into 6 well tissue culture plates (Corning) and 24 hours later were infected with 1.0 X 10^9 GC/well in a 6 well plate. After 48 hours, the supernatant was removed and 0.5% glutaraldehyde (Fisher) was added to the plates for 10 minutes. The plates were then washed with PBS and stained with 5-bromo-4-chloro- 3-indolyl b-D-galactoside (X-gal) for 3-4 hours at 37ºC. Following X-gal staining AAV-LacZ particle transduction was measured using a light microscope by counting the number of blue (AAV transduced) cells.

5.7 – In Vivo Studies Involving AAV Vectors expressing *lacZ*

5.7.1 – Immunization Schedule

For bio-distribution studies, groups of BALB/c (Charles River Laboratories, Saint-Constant, QC) mice were given a dose of $1x10^{11}$ GC of *lacZ* expressing AAV vectors by intravenous TV injection in 100µl volume. Control mice were injected in the same manner with phosphate buffered saline (PBS)

For gene delivery studies, groups of BALB/c mice were given doses of $1x10^{11}$ GC of *lacZ* expressing AAV vectors through alternate routes. To measure muscle gene delivery, IM injections were administered into the tibialis anterior in 50 µl volumes. To measure lung gene delivery, 50µl volumes were administered via IN route. To measure

liver gene delivery, 100µl volumes were administered via TV route (Figure 5.71). Control mice were given phosphate buffered saline (PBS) via the same route as for each group.

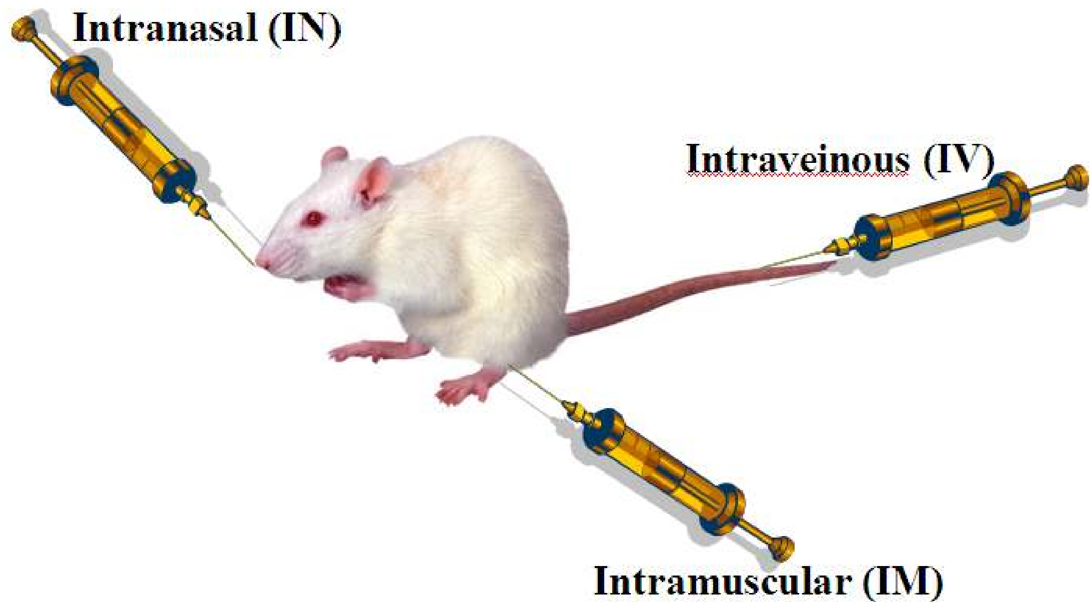


Figure 5.71 – Routes of Administration for AAV Vectors Expressing LacZ
Illustration depicting the various routes that were employed for characterization studies involving AAV vectors expressing LacZ.

5.7.2 – Biodistribution of Novel AAV Vectors

To measure biodistribution, different organs were collected 28 days post injection (d.p.i.) including the heart, liver, spleen, kidney, lungs, small intestine, large intestine, muscle, and pancreas and were analyzed by TaqMan® Probe-Based Gene Expression Analysis (Applied Biosystems, Carlsbad, CA, USA) for the amount of rAAV GC per cell. DNA was extracted from approximately 25mg of each tissue sample with QIAamp® DNA Mini Kit and TaqMan® amplification was performed as described previously [52]. A positive signal was determined when the number of GC was higher than 3 times of the amount obtained from PBS-injected control tissues. The number of transduced rAAV genomes in each organ was reported as number of GC/cell.

5.7.3 – Histological Analysis of Novel AAV Vectors

To measure muscle gene delivery, the tibialis anterior was harvested from mice 30 d.p.i. Harvested muscle tissue was embedded in Tissue-Tek® (optimal cutting temperature) O.C.T. Compound (Fisher) and was snap frozen in liquid nitrogen cooled isopentane. Lungs and tracheas were harvested from mice 64 days post IN administration to determine gene delivery to the lungs. The lungs were inflated with a 1:1 solution of PBS and Tissue-Tek® O.C.T. compound and embedded in Tissue-Tek® O.C.T. compound then frozen in ethanol cooled dry ice. Livers were harvested from mice 30 d.p.i. and embedded in Tissue-Tek® O.C.T. compound then frozen in ethanol cooled dry ice to quantify liver gene delivery. All harvested tissues were cut into 10µm thick sections using a Leica CM1850 Cryostat (Leica, Concord, ON) which were transferred to glass

slides. The slides were fixed with 1.6 % glutaraldehyde, incubated overnight with X-gal at 37ºC, lightly stained with eosin and dehydrated in 70-100% ethanol solutions. Sections were analysed on a light microscope.

5.7.4 – Serology of Novel AAV Vectors

BALB/c mice were injected IM with $1x10^{11}$ GC of porcine AAV vectors or an AAV2/5 control all expressing the *lacZ* transgene. 28 days post injection mice were trial bled via the saphenous vein. Mouse sera was obtained and inactivated at 56°C for 45 minutes. 2-fold serial dilutions using DMEM were performed for each sample starting with a 1:10 dilution. Samples were mixed with an equal volume of the appropriate AAV vector expressing *lacZ* and incubated at 37°C for 60 minutes. The mixture was then transferred onto subconfluent HEK 293T cells in 96 well flat bottom tissue culture plates (Corning) and incubated for 1.5 hours at 37°C with 5% CO_2. Control wells were infected with the same amount of *lacZ* expressing vectors in the presence or absence of non-immune serum. DMEM supplemented with 20% FBS was added to each well and the plates were incubated for 48 hours at 37°C with 5% CO_2. After incubation, cells were stained with X-gal and observed under a light microscope. The same procedure was repeated with using pooled human immune sera Carimune® NF, Nanofiltered Immune Globulin Intravenous (Human) Lyophilized Preparation (CSL Behring, King of Prussia, PA, USA). The NAb titre was reported as the highest serum dilution that inhibited AAV-LacZ particle transduction (X-gal expression) by >50% compared with the control.

5.8 – In Vivo Studies Involving AAV Vectors expressing HA

5.8.1 – Immunization and Challenge Schedule

To determine immunological profiles of the novel AAVs, groups of 6-8 week old BALB/c mice were injected IM with a dose of 2.5×10^{10} GC of each HA expressing AAV vector in 50µl into the left hind limb. Injections dates were staggered as to accommodate sampling at days 10, 20, 30, and 40 post vaccination.

For protection studies, groups of 6-8 week old BALB/c mice were immunized with a dose of 2.5×10^{10} GC of each HA expressing AAV vector to via IM injection of 50µl into the left hind limb. Control mice were either injected IM with 2.5×10^{10} GC in 50µl of AAV vectors expressing LacZ or IM with 50µl of PBS, both in the same manner as described above. Avian influenza H5N1 strain A/Hanoi/30408/2005 (Hanoi 2005) was kindly provided by Q. Mai Le and T. Hien Nguyen, National Institute of Hygiene and Epidemiology, Hanoi, Vietnam. On day 25 post injection, mice were trial bled via the saphenous vein for all samples and serum was collected. On 28 d.p.i. mice were challenged by IN inoculation with 100LD50 of Hanoi 2005 virus in 50µl of virus diluent (MEM, 0.3%BSA, antibiotics).

5.8.2 – Characterization of T Cell Responses to Novel AAVs

Mice were sacrificed on days 10, 20, 30 and 40 post vaccination, and spleens were harvested to perform enzyme-linked immunosorbent spot (ELISPOT) assays along with Fluorescent Activated Cell Sorting (FACS).

5.8.2.1 – Separation of Splenocytes

To isolate splenocytes, harvested spleens were ground against a screen mesh in L-15 medium (Gibco), then were passed through a 40μm cell strainer (BD Bioscience) and centrifuged at 485g for 7 minutes. The cell pellet was re-suspended in 10ml of L-15 and counted using a haemocytometer (Fisher).

5.8.2.2 – Resuspension of Lyophilized Peptide Libraries for Detection of HA

Lyophilized peptide libraries (Mimitopes, Clayton, Victoria, Australia) were acquired containing 15 amino acid long sequences with 10 amino acid long overlaps spanning the complete HA protein of H5N1. The lyophilized peptides were resuspended in dimethyl sulfoxide (DMSO) to a final concentration of 100μg/μl, aliquoted, and stored at -80°C for long term storage. A matrix of peptide pools was created for efficient screening of the entire peptide library and a pool of 27 peptides was made for HA.

5.8.2.3 – ELISPOT Procedure

ELISPOT assays were performed using the Mouse IFN-γ ELISPOT Set (BD Biosciences) according to the manufacturer's recommended protocols. Immobilon®-P 96-well flat bottom microtitre plates (PVDF membrane, Millipore) were coated with purified anti-mouse IFN-γ antibody (BD Biosciences) diluted in PBS at 4°C overnight. Afterwards, each plate was blocked for 3 hours using RPMI medium 1640 supplemented with 10% FBS, and 1% penicillin/streptomycin. Splenocytes were added at

concentration of 5 x 10^5 cells per well and plates were incubated overnight at 37°C in a humid atmosphere containing 5% CO_2. The next day, plates were washed 2X with water, 3X with Solution I (PBS and 0.1% tween-20) and incubated with biotinylated anti-mouse IFN-γ for approximately 2 hours at room temperature. Following 2 hour incubation, plates were washed 3X with Solution I and then incubated with streptavidin-HRP. Cells secreting IFN-γ were visualized as spots through use of the AEC Substrate Reagent Set (BD Biosciences) on the PVDF membrane. Spots were counted using an AID ELISPOT Reader (Cell Technology, Colombia, MD, USA) and reported as spot forming units (SFU) per 1 million cells.

5.8.3 – Characterization of B Cell Responses to Novel AAVs

Mouse sera collected 25 d.p.i. from the challenge mice was used for Hemagglutination Inhibition (HI) Assays and for Neutralization Assays. Mouse sera were treated overnight at 37°C with the receptor destroying enzyme (RDE) (Sigma-Aldrich) and then inactivated at 56°C for 45 min the next day before use for antibody assays.

5.8.3.1 - Hemagglutination Inhibition Assays

2-fold serial dilutions were performed on RDE-treated mouse sera starting with a 1:10 dilution. 50µl of sera were added to each well in a 96 well V-bottom microtitre plate (Corning). Four hemeagglutinating doses (AD) of the Hanoi 2005 virus were added to each well and the plate was incubated at room temperature for 1 hour. After the incubation, 50µl of 0.5% turkey, 0.5% horse, or 0.5% guinea pig red blood cells were added to each well and the assay was incubated at room temperature for up to 1 hour.

The HI titre was scored as the highest dilution where red blood cell agglutination did not occur and the data were reported as the reciprocal of this dilution.

5.8.3.2 – Neutralizing Antibody Assays

2-fold serial dilutions were performed on RDE-treated mouse sera (prepared in virus diluent) starting with a 1:10 dilution and were mixed with an equal volume of the Hanoi 2005 virus which was 100 plaque forming units (pfu) per well. The mixture was incubated at 37°C for 1 hour, then transferred onto subconfluent MDCK cells in 96 well flat bottom tissue culture plates and left at room temperature for 5-10 minutes. 2.0µg/ml of TPCK-trypsin (Sigma-Aldrich) was supplemented into 100µl of virus diluent and then added to each well of the plates which were then incubated at 37°C in humid atmosphere containing 5% CO_2 for 48 hours. Following incubation, MDCK cells were analyzed under a light microscope for the presence of cyopathic effects (CPE). The highest dilution of sera not displaying CPE was recorded as positive for neutralizing antibodies and neutralization titres were reported as the reciprocal of this dilution.

5.9 – Animal Containment and Scoring During Studies

Work involving administration of AAV Vectors was performed in Biological Safety Level 2 (BSL-2) laboratories at the National Microbiology Laboratory (NML) of the Public Health Agency of Canada (PHAC). Infectious work involving mouse challenge with Hanoi 2005 was carried out in BSL-4 at the NML/PHAC. Mice were housed in filtered cages and anaesthetized with Aerrane® Isoflurane (Baxter Corporation,

Mississauga, ON). All animal work was first approved by the in house animal care committee, according to guidelines set by the Canadian Council on Animal Care. Mice were monitored for weight loss and signs of disease over a period of 15-20 days following infection. The mice were scored on a scale of 0-3: 0-no symptoms, 1-ruffled fur, slowing activity, loss of body conditions, 2- hind limb paralysis, laboured breathing, hunched posture, 3-death. All animal procedures were approved by the Institutional Animal Care Committee (IACC) at the NML/PHAC, according to the guidelines of the Canadian Council on Animal Care.

6.0 – Results

6.1 – Identification of Novel AAV Sequences from Porcine Tissues via PCR

Given that AAV has the capacity to integrate its genome into the host cell during a latent infection, the use of PCR techniques was employed to isolate novel AAV sequences from genomic porcine DNA similar to previous studies by Gao et al. [77] and Mori et al. [80]. Different porcine tissues including lung, liver, heart, spleen, and gut were obtained from industrial slaughterhouses and private rural farms. DNA was isolated from porcine tissues and screened by PCR for the presence of novel AAV sequences. Primers specific for conserved regions of the AAV genome generated at least four distinct AAV sequences from porcine tissues that were termed AAVpo1, AAVpo4, AAVpo5 and AAVpo6. AAVpo1 was isolated from spleen tissue while AAVpo4, AAVpo5 and AAVpo6 were all isolated from intestinal tissues. PCR amplifications were also performed to isolate 1.64 kb sequences spanning the AAV *rep* and *cap* genes in addition to a 1.4 kb sequences that overlap with the 1.64 kb fragments covering the 3′ end of the *cap* gene. The entire cap genes for AAVpo1, po4, po5, and po6 were isolated from the porcine tissues and sequenced. Sequences for the newly isolated porcine AAV *cap* genes were aligned with sequences from known AAV serotypes using the Lasergene® MegAlign software (DNASTAR Inc, Madison, WI, USA). Phylogenetic trees based on sequence homology were generated (Figure 6.1) and sequence distances were also measured (Table 6.1). AAVpo1 displayed 89.8% sequence homology with AAVpo5 and both were closely related to AAV5. AAVpo4 showed 90.1% sequence homology with AAVpo6 and both were related to AAV2.

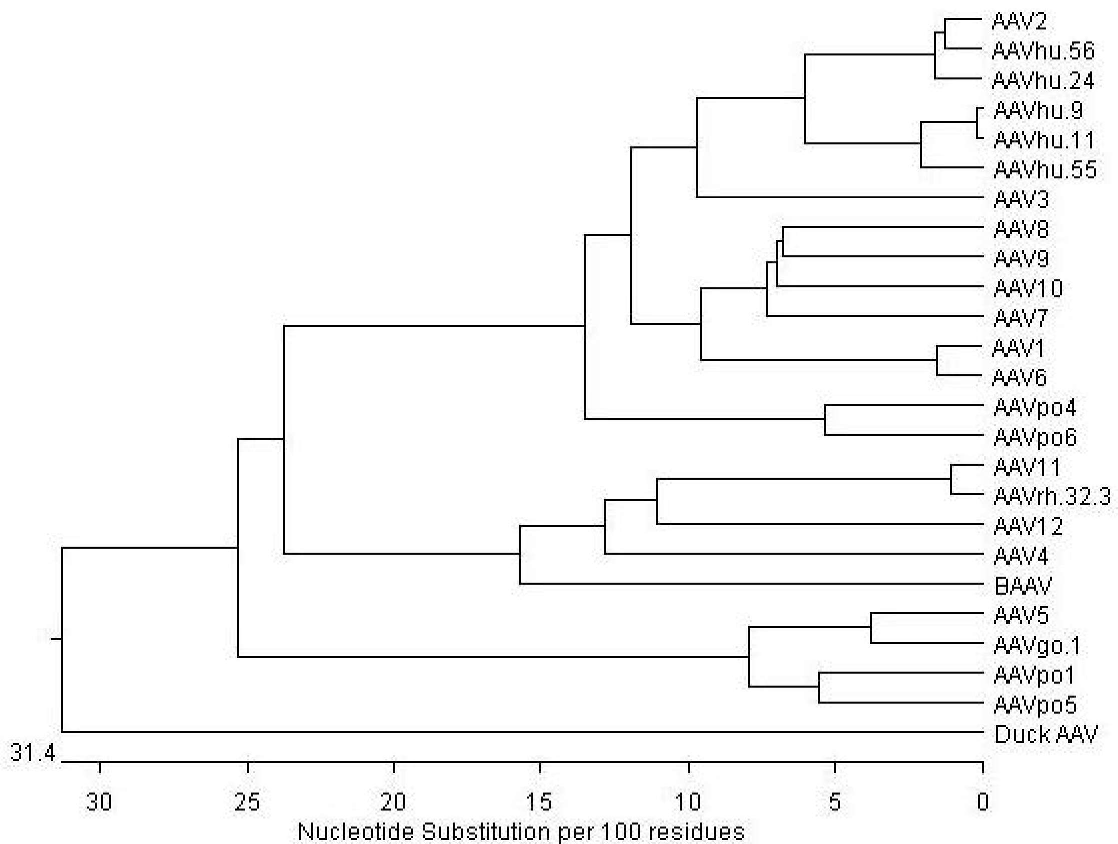


Figure 6.1 – Phylogenetic Tree Comparison of Novel Porcine AAVs
Phylogenetic tree depicting sequence homology based on nucleotide substitutions for *cap* genes of known AAV serotypes and novel AAV serotypes isolated from porcine tissues generated using the Lasergene® MegAlign software.

Table 6.1 – AAV Serotype Percent Identities Based on Sequence Homology

Percent Identity (upper triangle); Divergence (lower triangle)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25		
1		79.1	79.5	66.7	62.8	97.0	83.9	83.1	82.4	83.3	70.0	66.0	69.7	79.8	80.0	79.6	80.4	78.9	62.8	60.0	63.5	65.0	77.4	64.0	77.1	**1**	AAV1
2	24.6		81.2	65.1	64.2	79.6	78.4	79.2	78.9	80.5	65.7	64.6	65.8	88.3	88.4	96.8	90.0	97.5	63.6	58.9	61.8	64.8	79.8	64.2	80.7	**2**	AAV2
3	24.1	21.7		66.2	63.0	79.7	80.1	80.0	79.0	79.9	66.2	65.3	66.3	84.8	84.8	81.2	85.0	80.8	63.0	59.9	61.7	63.8	76.3	64.3	76.4	**3**	AAV3
4	44.2	47.0	45.2		60.8	66.9	67.2	67.7	66.8	67.7	78.5	78.1	78.6	65.9	66.0	65.4	65.7	65.2	60.7	56.5	74.5	61.3	66.1	61.1	65.7	**4**	AAV4
5	51.5	48.9	51.4	55.7		62.6	64.0	64.8	63.5	63.6	59.5	59.5	59.4	63.6	63.6	64.3	63.8	64.0	92.8	57.2	63.3	84.8	68.3	85.4	67.5	**5**	AAV5
6	3.1	23.9	23.9	43.7	52.0		83.5	82.7	82.1	83.1	69.7	65.9	69.4	79.9	80.2	79.9	80.4	79.4	62.6	59.9	63.5	64.8	77.6	63.8	77.0	**6**	AAV6
7	18.2	25.6	23.4	43.3	49.2	18.8		86.2	85.3	87.7	69.7	66.1	69.7	79.1	79.2	78.1	79.6	77.9	63.7	59.8	63.3	64.4	77.5	65.2	77.4	**7**	AAV7
8	19.2	24.4	23.4	42.4	47.6	19.7	15.4		87.7	88.7	68.9	66.2	68.5	79.7	79.9	79.1	79.8	78.7	64.9	60.3	62.7	66.1	77.6	66.1	78.1	**8**	AAV8
9	20.1	24.8	24.9	43.9	50.2	20.5	16.5	13.5		86.0	68.6	65.6	68.8	78.4	78.6	78.5	78.9	78.7	63.5	59.5	62.1	64.8	76.7	64.6	76.5	**9**	AAV9
10	18.9	22.7	23.6	42.4	50.0	19.2	13.5	12.3	15.6		70.6	66.4	70.2	80.0	80.2	80.8	80.6	80.1	64.1	60.4	63.2	65.4	78.0	65.8	78.9	**10**	AAV10
11	38.4	46.0	45.1	25.4	58.6	38.9	38.8	40.4	40.8	37.4		80.8	97.9	66.4	66.7	65.5	66.6	65.2	59.8	58.1	74.5	60.9	63.7	60.0	64.1	**11**	AAV11
12	45.5	48.0	46.7	26.0	58.8	45.7	45.4	45.2	46.4	44.6	22.4		81.2	65.6	65.7	65.5	65.7	64.8	59.5	57.6	73.8	60.0	63.6	59.7	63.0	**12**	AAV12
13	38.9	45.8	45.1	25.4	58.9	39.5	38.9	41.0	40.4	38.1	2.2	21.9		66.5	66.8	65.8	66.7	65.5	60.3	58.3	74.8	61.1	63.4	60.0	63.8	**13**	AAVrh.32.33
14	23.7	12.7	17.1	45.6	50.1	23.4	24.6	23.7	25.6	23.5	44.6	46.3	44.5		99.6	88.1	95.9	87.8	63.4	59.7	62.0	64.2	77.9	64.5	79.0	**14**	AAVhu.9
15	23.4	12.6	17.0	45.4	50.1	23.1	24.4	23.5	25.3	23.1	44.1	45.9	44.0	0.4		88.2	96.1	88.0	63.4	59.7	62.0	64.4	78.0	64.5	79.1	**15**	AAVhu.11
16	23.9	3.3	21.7	46.5	48.7	23.4	26.0	24.6	25.4	22.3	46.3	46.4	45.8	13.0	12.9		89.3	96.9	64.1	58.9	62.4	64.9	80.0	64.4	81.2	**16**	AAVhu.24
17	22.8	10.7	16.9	45.9	49.6	22.8	24.0	23.7	24.9	22.6	44.3	46.0	44.1	4.3	4.1	11.6		89.6	63.8	60.0	62.5	64.5	77.8	64.7	78.6	**17**	AAVhu.55
18	24.8	2.5	22.4	46.9	49.2	24.2	26.3	25.1	25.1	23.3	47.0	47.6	46.4	13.3	13.1	3.2	11.3		63.5	59.0	62.0	64.5	79.8	64.1	80.6	**18**	AAVhu.56
19	51.6	49.9	51.3	55.8	7.5	52.1	49.9	47.5	50.2	49.0	58.1	58.6	57.2	50.4	50.5	49.0	49.5	50.3		57.1	63.8	86.3	67.8	86.4	66.6	**19**	AAVgo.1
20	57.7	60.1	57.9	65.6	64.7	57.8	58.1	57.0	58.6	56.8	62.1	63.2	61.7	58.1	58.2	60.1	57.5	59.9	64.3		56.2	57.4	58.4	57.8	58.5	**20**	Duck AAV.seq
21	50.2	53.6	53.8	31.2	50.6	50.1	50.6	51.7	52.8	50.7	31.2	32.4	30.8	53.1	53.1	52.2	52.1	53.1	49.8	66.4		62.1	64.9	61.7	65.2	**21**	BAAV
22	47.2	47.6	49.7	54.6	17.1	47.5	48.4	45.2	47.7	46.5	55.6	57.5	55.1	48.8	48.5	47.4	48.3	48.2	15.2	63.6	53.0		68.9	89.8	67.6	**22**	AAVpo1
23	27.1	23.7	28.7	45.2	41.3	26.9	26.9	26.7	28.2	26.2	49.9	50.0	50.4	26.4	26.2	23.5	26.5	23.8	42.1	61.4	47.5	40.3		69.1	90.1	**23**	AAVpo4
24	49.3	48.8	48.8	55.0	16.3	49.8	47.0	45.2	48.2	45.9	57.6	58.3	57.5	48.3	48.4	48.5	48.0	49.2	15.1	62.7	54.1	11.1	39.9		68.2	**24**	AAVpo5
25	27.5	22.6	28.7	46.1	42.8	27.7	27.1	26.0	28.3	24.9	49.1	51.4	49.6	24.9	24.7	21.8	25.4	22.6	44.4	61.2	46.9	42.6	10.6	41.4		**25**	AAVpo6
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**		

6.2 – Production of Hybrid Porcine AAV Vectors Expressing LacZ

Various studies have shown that the AAV2 *rep* gene can be psuedotyped with other AAV *cap* genes allowing for efficient production of functional AAV particles containing the capsid protein of choice. Successful production of hybrid porcine AAV vectors was achieved via a triple transfection system using HEK 293T cells. The three plasmids included the *trans* packaging plasmid encoding for the AAV2 *rep* and porcine AAV *cap* genes (po1, po4, po5, and po6), along with the LacZ encoding *cis* plasmids and the Ad gene encoding helper plasmid (pAd-DeltaF6) needed to drive replication.

Recombinant AAV vectors were purified by CsCl gradient purification and protein isolates were run on an SDS-PAGE gel under reducing conditions visualized by Western blot. Mouse monoclonal antibodies against AAV2 VP1, VP2 and VP3 were used for the

porcine AAV serotypes. Western blot produced a distinct pattern of VP1, VP2, and VP3 capsid proteins (Figure 6.2a) verifying proper viral protein expression. Electron microscopy (EM) was also performed on the newly produced porcine AAV serotypes to determine whether porcine AAV particles are assembling and being produced during virus production. EM studies revealed that the porcine AAV particles were comparable in shape and size to AAV2/5 with a diameter of 20-25 nm (Figure 6.2b). It should be noted that at the time of characterization AAV2/po6 had not yet been isolated and thus was not present for studies involving *lacZ*.

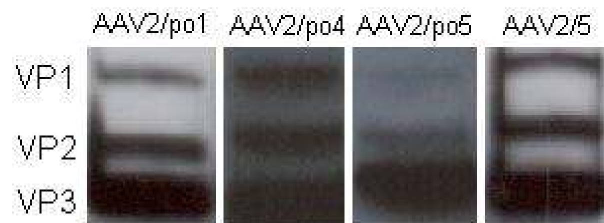


Figure 6.2a - Western blots of Porcine AAV Capsid Proteins
Capsid proteins for AAV2/po1, AAV2/po4, AAV2/po5 and AAV2/5 were obtained from transfected HEK 293T cells. Samples were separated via 10% SDS-PAGE and later transferred to a PVDF membrane. Anti-VP1, -VP2, and -VP3 mouse monoclonal antibodies were used as the primary antibody and goat anti-mouse (HRP)-conjugated antibody as the secondary antibody.

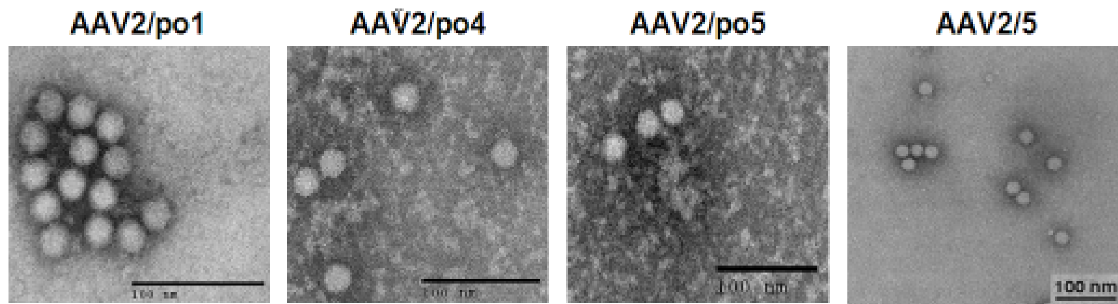


Figure 6.2b – Electron Microscopy of Porcine AAV Serotypes
Electron microscopy of hybrid porcine AAV2/po1, AAV2/po4, and AAV2/po5 serotypes along with AAV2/5 each encoding the lacZ transgene. Particles are at 100,000X magnification and were isolated from transfected HEK 293T cells purified by CsCl gradients.

6.3 – Transduction Efficiency of Hybrid Porcine AAV Vectors *In Vitro*

To determine the transduction efficiency (TE) of the porcine AAVs, various cell lines were infected with porcine vectors expressing LacZ and AAV2/5 as a control. The porcine AAV vectors were able to transduce all cell lines tested and performed with equal frequency as the AAV2/5 control. AAV2/po1 was able to transduce HepG2 (Human liver cells), MDCK (Canine Kidney cells), VRIBL-E1 (Porcine fetal retina cells), HEK 293T (Human embryonic kidney cells) and RAW 264.7 (Mouse macrophage cells) with higher efficiency than AAV2/5, having the highest TE in VRIBL-E1 and lowest TE in A549 cells (Human lung cells). AAV2/po4 was able to transduce VRIBL-E1 and HEK 293T cells with higher efficiency than AAV2/5, also having the highest TE in VRIBL-E1 and lowest TE in A549 cells. AAV2/po5 was able to transduce VRIBL-E1 and Vero E6 (African green monkey kidney epithelial) cells with higher efficiency than AAV2/5 and also had the highest TE in VRIBL-E1 and the lowest TE in A549 cells (Figure 6.3).

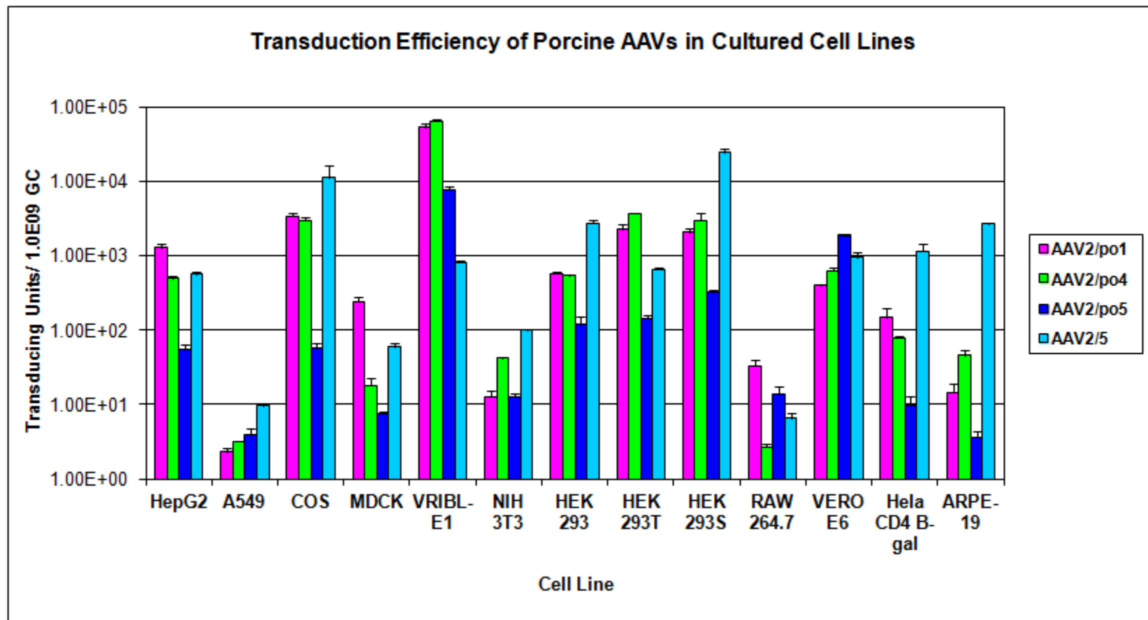


Figure 6.3 - Transduction Efficiency of Porcine AAV Vector *In Vitro*
Subconfluent cells lines were infected with 1.0 x 10^9GC/well of AAV vectors expressing LacZ. Infection proceeded for 48 hours after which cells were fixed with 0.5% glutaraldehyde for 10 minutes and stained with X-gal at 37°C for 3-4 hours. Cells were viewed under a light microscope and transduced (blue) cells were recorded.

6.4 – Biodistribution and Histology of Porcine AAV Vectors Expressing LacZ

The biodistribution and histology of the novel porcine AAV vectors was studied *in vivo*. AAV vectors expressing the LacZ transgene were administered into the muscle (IM), liver (IV) or lung (IN) of mice at a dose of 1.0x10^{11} GC per mouse and tissues were harvested 28 days post infection. The biodistribution of all AAV serotypes was widespread and comparable to the AAV2/5-LacZ control vector. The AAV2/po4-LacZ vector was found to be present in high concentration within the liver tissues of mice and this was also verified by histology of liver tissues (Figure 6.4).

Histology of mouse organs revealed that both AAV2/po1 and AAV2/po4 transduced muscle tissue most efficiently and AAV2/po4 transduced liver most efficiently, whereas AAV2/po5 did not transduce either tissue efficiently. All three vectors did not transduce lung tissue efficiently and had lower levels than the AAV2/5 control (Figure 6.4).

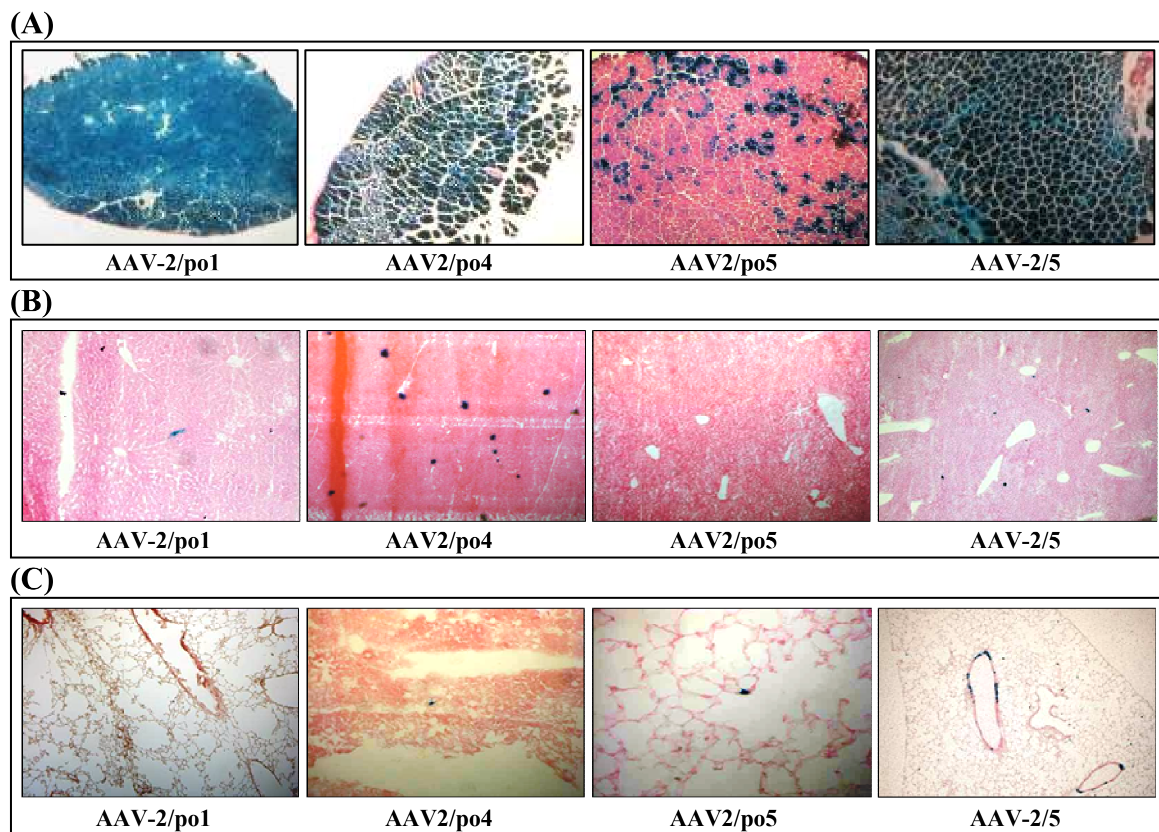


Figure 6.4 – Histology of Porcine AAV Vectors in BALB/c Mice
BALB/c mice were vaccinated either IM (tibialis anterior), IV (tail vein), or IN with 1.0×10^{11} GC of AAV vectors expressing the LacZ transgene. Tissues were harvested 28 d.p.i., frozen with OCT, and sections were cut using a cryostat. Sections were fixed and stained with X-gal, then visualized 24 h later. Blue colour indicates tissue transduction in muscle (A), liver (B), and lung (C).

6.5 – Cross Neutralization and Seroprevalence of Novel AAV Vectors

Neutralization assays were performed on each of the *lacZ* expressing AAV vectors using either sera from AAV vaccinated mice, or pooled human Ig (CSL Behring). The sera from AAV-LacZ vaccinated mice were used for cross-neutralization studies and the pooled human Ig was used to measure seroprevalence of the novel serotypes. Antiserum from AAV2/po1, -po4, -po5, -po6 and AAV2/5 vaccinated mice had neutralizing antibody titres of 1:640, 1:640, 1:50 and 1:5120 against themselves, respectively (Table 6.4). In contrast, all porcine AAV antiserum had undetectable neutralizing antibodies against AAV2/5 and vice versa.

To address seroprevalence in humans, the porcine AAV vectors along with the AAV2/5 control were incubated with pooled human Ig at various dilutions. Neutralization was not detected for any of the porcine serotypes with undiluted human Ig at 12 mg/ml whereas 50% of the AAV2/5 serotype was neutralized with 0.1875 mg/ml of pooled human Ig (Table 6.4).

Table 6.5 – Cross-Neutralization and Seroprevalence of Porcine AAVs

	Antisera				
Vector	**AAV2/po1**	**AAV2/po4**	**AAV2/po5**	**AAV2/5**	**Pooled Human IgG**
AAV2/po1	1:640,	<1:20	<1:20	<1:20	>12
AAV2/po4	<1:20	1:640,	<1:20	<1:20	>12
AAV2/po5	<1:20	<1:20	1:40	<1:20	>12
AAV2/5	<1:20	<1:20	<1:20	1:5120	0.1875

6.6 – Production of Hybrid Porcine AAV Vectors Expressing H5N1-HA

After physical characterization, measurement of biodistribution and histology of the novel porcine AAVs, the immune response to these vectors were analyzed *in vivo*. The HA antigen from the highly pathogenic A/Hanoi/30408/2005 (H5N1) strain of influenza virus was chosen as the transgene for the novel AAV vectors to express in order to measure their immune profiles. The porcine AAV vectors could then be identified as being suitable for either gene or vaccine candidates based on their level of immunogenicity.

Production of hybrid porcine AAV vectors was achieved via a triple transfection system using HEK 293T cells as described previously, with the only difference being that the *cis* plasmid used for transfection encoded an HA transgene from Hanoi 2005. AAV vectors expressing HA were purified by CsCl gradient purification and production of HA proteins was measured by SDS-PAGE and visualized by Western blot (Figure 6.6).

Figure 6.6 - Western blots of H5N1 expression from porcine AAVs
Protein samples were obtained from subconfluent HEK 293T cells that were infected at a M.O.I of 60,000 with each AAV expressing HA vector for 72 hours. Samples were separated via 10% SDS-PAGE and later transferred to a PVDF membrane. Mouse monoclonal antibodies against H5N1-HA were used as the primary antibody along with a goat anti-mouse (HRP)-conjugated secondary antibody.

6.7 – Characterization of T Cell Responses to Novel AAVs *In Vitro*

6.7.1 – T Cell Response Kinetics to Novel AAVs

The presence or absence of cell-mediated immune responses following vaccination with novel AAV serotypes will help in determining their potential use as either gene therapy or vaccine candidates. To investigate the immunological profiles of the novel porcine AAV vectors, groups of BALB/c mice were vaccinated IM with AAV2/po1-H5N1-HA, AAV2/po4-H5N1-HA, AAV2/po5-H5N1-HA, AAV2/po6-H5N1-HA, AAV2/8-H5N1-HA or AAV2/rh32.33-H5N1-HA at a dose of $2.5x10^{10}$ GC/mouse. Mice were then sacrificed and splenocytes isolated on days 10, 20, 30 and 40 post immunization. Mouse T cell responses to the porcine AAV vectors were evaluated by ELISpot-IFN-γ assay which was carried out on the harvested splenocytes from each time point post-immunization. Splenocytes were re-stimulated *ex vivo* with 27 pools of overlapping peptides corresponding to the HA protein of Hanoi 2005 and T cell responses were visualized through detection of spot forming cells secreting IFN-γ. On days 10, 20, 30 and 40 post vaccination, T cell responses were highest in mice vaccinated with AAV2/8-H5N1-HA, AAV2/rh32.33-H5N1-HA, AAV2/rh32.33-H5N1-HA and AAV2/po6-H5N1-HA, 1592.70 , 6808.70, 6724.67 and 7284.67 SFU/$1x10^6$ cells respectively (Figure 6.71). T cells responses were the lowest in mice vaccinated with AAV2/po5-H5N1-HA on days 10, 20, and 30, 310.70, 776.00, 2500.70 SFU/$1x10^6$ cells respectively. On day 40, T cell responses were the lowest in mice vaccinated with AAV2/po4-H5N1-HA at 4496.67 SFU/$1x10^6$ cells (Figure 6.7).

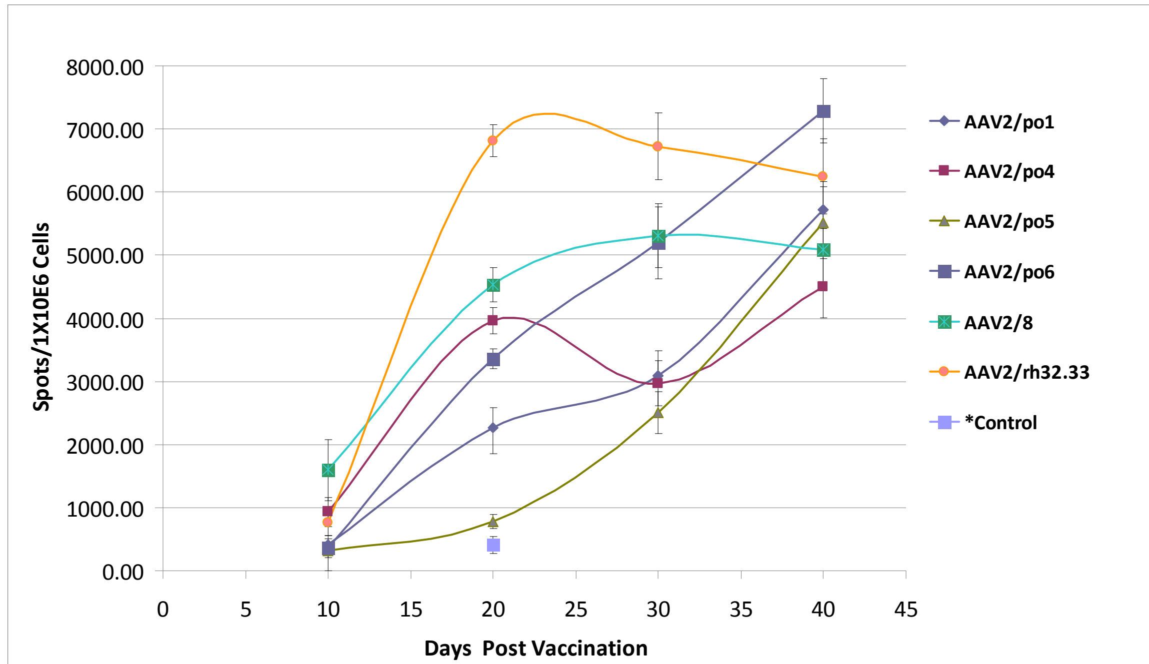
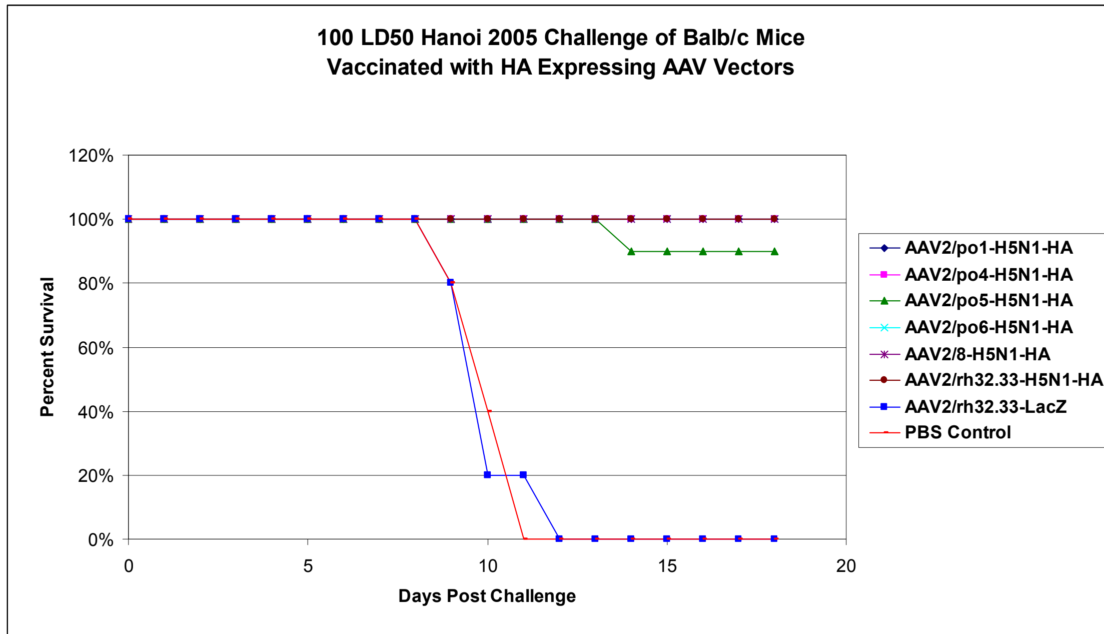


Figure 6.71 - Pooled H5N1-HA Peptide Responses for IFN-γ
Pooled peptide responses for groups of 4 BALB/c mice vaccinated IM with $2.5x10^{10}$ GC of rAAV vectors expressing the HA from Hanoi 2005. ELISPOTS were performed on splenocytes harvested from mice 10, 20, 30 and 40 days post-vaccination. Splenocytes were stimulated with 27 different pools of 15mer peptides spanning the entire HA protein. Responses were visualized as spots representing IFN-γ production by cytotoxic T cells.

6.7.2 – Protection Studies of Novel AAV Vectors Following Lethal Challenge

To further characterize immune responses and potential use of the porcine AAV vectors as vaccine candidates, a lethal challenge with Hanoi 2005 was carried out in vaccinated BALB/c mice. Groups of 10 BALB/c mice were vaccinated IM with the corresponding HA expressing AAV vector and two groups of 5 BALB/c mice were used for controls. Mice were challenged IN with a $100LD_{50}$ dose of Hanoi 2005 in BSL-4 (Ami Patel, Alex Bello) on day 28 post-vaccination. All mice vaccinated with AAV vectors expressing HA had 100% percent survival except in the AAV2/po5-H5N1-HA group which had 90% survival, and this experiment was repeated to confirm our findings. All surviving animals did not show any signs of disease and had minimal changes in weight. Mice in both control groups did not survive and the first animals succumbed to disease at day 9 post challenge. The control animals began to display visible signs of disease on day 7 post challenge and weight loss occurred on day 3 post challenge (Figure 6.72).

(A)



(B)

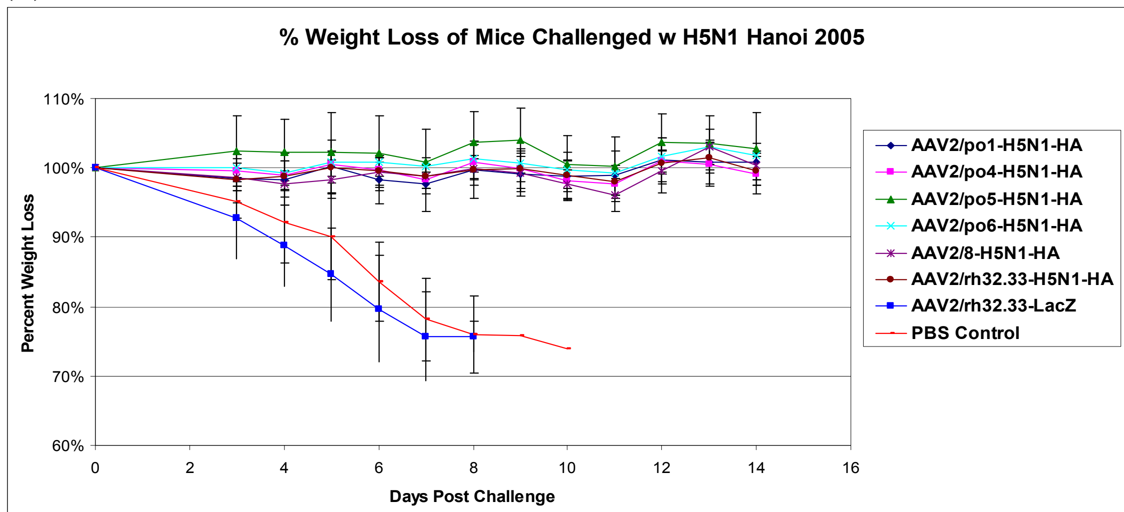


Figure 6.72 – Protection of Vaccinated Mice Challenged with Hanoi 2005
Groups of 10 BALB/c mice were vaccinated with 2.5×10^{10} GC of rAAV expressing H5N1-HA. Groups of 5 mice were vaccinated with an AAV expressing LacZ or PBS as controls. Mice were challenged with Hanoi 2005 28 days post-vaccination. Mice were observed for (A) survival and (B) weight loss.

6.8 - Characterization of B Cell Responses to Novel AAVs *In Vitro*

6.8.1 – Antibody Responses to Novel AAVs

To further our understanding of the novel AAV serotypes, humoral immune responses were also evaluated in mice. Antibody responses were evaluated through the detection of HI titres following vaccination with AAV. Groups of 10 BALB/c mice were vaccinated with 2.5×10^{10} GC of AAV2/po1-H5N1-HA, AAV2/po4-H5N1-HA, AAV2/po5-H5N1-HA, AAV2/po6-H5N1-HA, AAV2/8-H5N1-HA or AAV2/rh32.33-H5N1-HA and serum was collected from the animals 25 days post-vaccination. Serum samples were treated with RDE at 37°C overnight and complement was inactivated at 56°C for 45 minutes. Horse red blood cells were used to assay HI antibody titres and results were scored as the reciprocal of the highest dilution which did not agglutinate red blood cells. HI levels were detected against the Hanoi 2005 virus before challenge for each AAV HA vector. Reciprocal titres of 104, 184, 152, 136, 136, and 140 were detected for AAV2/po1-H5N1-HA, AAV2/po4-H5N1-HA, AAV2/po5-H5N1-HA, AAV2/po6-H5N1-HA, AAV2/8-H5N1-HA and AAV2/rh32.33-H5N1-HA respectively (Figure 6.81).

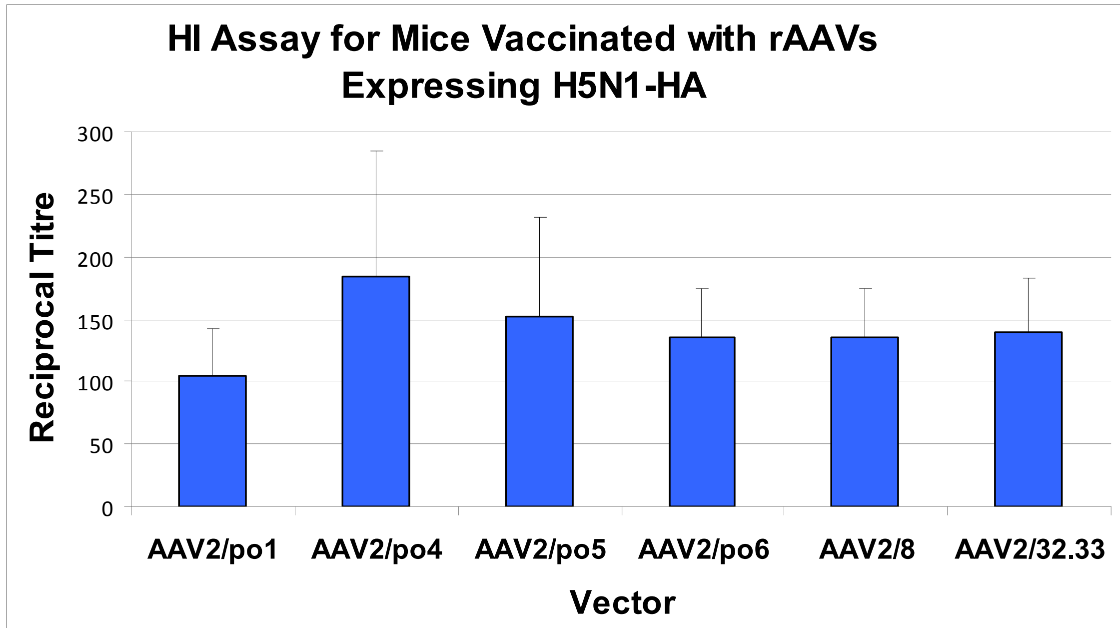


Figure 6.81 – HI titres against Hanoi 2005 in BALB/c Mice
Serum was collected 25 days post injections from BALB/c mice vaccinated IM with AAV vectors expressing H5N1-HA. Samples were pre-treated overnight with RDE and inactivated the following morning. Serial dilutions were performed and 4 hemagglutinating doses of Hanoi 2005 virus were added to each well. The serum-virus mixture was incubated with 0.5% horse RBCs and HI titres were reported as the reciprocal of the highest dilution of serum which prevented agglutination.

7.0 – Discussion

7.1 – Isolation and Production of Novel Porcine AAV Serotypes

The majority of AAV vectors are based on the human AAV2 serotype. AAV2 is highly seroprevalent and a large portion of the human population already have neutralizing antibodies to this serotype [41]. Pre-existing immunity can interfere with gene therapy or vaccine applications by hindering transgene expression and transduction efficiency leading to ineffective treatments following vector administration. As a result, it is important to identify novel AAV serotypes that are of low seroprevalence that can offer better alternatives for gene therapy and vaccine development. Studies have shown that human and porcine tissues are closely related and porcine tissues have been successfully implemented in pig-to-human xenotransplantation [76]. For these reasons, our lab hypothesized that a high histocompatibility between porcine and human tissues would result in porcine AAVs having good transduction efficiency and high safety profile for therapeutic applications in humans.

The screening of porcine tissues using primers based on conserved regions of the AAV *cap* gene resulted in the isolation of 4 novel porcine AAV serotypes AAVpo1, -po4, -po5 and -po6. The majority of these serotypes were isolated from porcine intestinal tissues and these findings may coincide with the fecal-oral route of transmission for AAV. An interesting observation was that sequences identical to the human AAV2 serotype were also present in the porcine tissue samples and that the porcine AAV serotypes AAVpo4 and AAVpo6 were closely related to AAV2. This may be an indication for zoonotic

transmission and possibly co-evolution of specific porcine AAV serotypes and those found in humans further supporting the model of compatibility between the two species. This method of isolation has proven to be an efficient tool for screening porcine tissues and has also been effectively used for screening of NHPs by other groups [77, 80].

Complete sequences of the *cap* genes for each porcine AAV were used to generate functional hybrid AAV2/po1, AAV2/po4, AAV2/po5 and AAV2/po6 particles expressing the *lacZ* transgene. Vector production was carried out by pseudotyping with the AAV2 *rep* gene during triple transfection with *cis*, *trans*, and helper plasmids. Porcine AAV vectors were purified using CsCl gradient purification which produced relatively high yields of up to 1.0×10^{13} GC/ml of each vector. Western blot analysis of the hybrid vectors revealed proper expression of VP1, VP2 and VP3 proteins in comparison to AAV2/5. EM was also carried out on the porcine AAV vectors and verified that particles of 20-25 nm in size were assembling and being produced during virus production. Based on these findings it was concluded that the porcine AAV vectors were viable and ready for *in vitro* and *in vivo* studies.

7.2 – Transduction Efficiency and Tropisms of Novel AAV Vectors Expressing l*acZ*

In the current context, AAV vectors have mainly been viewed as vehicles for gene therapy and for these reasons attributes of low seroprevalence, stable gene expression and high levels of transduction are the most desirable. The target tissue and tropism of an AAV vector are also important factors in treating disorders limited to a specific organ or

area of the body. The porcine AAV serotypes that were produced were able to transduce all cell lines that were tested. In most cases the TE of AAV2/po1 and AAV2/po4 was comparable to that of the control AAV2/5 vector however it was noticeably higher in the VRIBL-E1 cells line. The AAV2/po5 vector had the lowest TE overall but performed better than the control AAV2/5 vector in the VRIBL-E1 and Vero E6 cell lines most likely due to tropism or receptor specificity. Since the porcine vectors all had high TE in the VRIBL-E1 cell line it was proposed that these vectors may function well for gene therapy studies involving retinal diseases such as Leber congenital amaurosis [81].

In vivo studies demonstrated that the porcine AAV vectors were also efficient at transducing tissues in BALB/c mice and had tropisms for muscle and liver tissues. AAV2/po1 and AAV2/po4 both transduced muscle tissue efficiently and AAV2/po4 was better at transducing the liver in comparison to the AAV2/5 control. AAV2/po5 was not as effective at transducing any of the tissues tested by comparison to the other vectors. The AAV2/5 control was more effective at transducing lung tissue than all three porcine vectors tested. Effective transduction of muscle tissue by AAVp2/po1 along with reduced transduction of other tissues could perhaps make AAV2/po1 an attractive gene transfer vehicle for systemic delivery and may help the treatment of myopathies such as Duchenne muscular dystrophy [82]. AAV2/po4 was not only able to transduce muscle tissue efficiently but liver tissue as well and in higher levels than the AAV2/5 control. Higher levels of transduction in the liver may make AAV2/po4 suitable for liver gene therapy in treating ailments such Haemophilia B [83] or Glycogen storage disease [84].

The differences observed between the porcine vectors can be attributed to sequence variation as closely related serotypes may still have different tissue tropisms.

The purpose of looking for AAVs in porcine tissues was to find potential serotypes of low seroprevalence that would be compatible for human applications. The lack of cross-neutralization between the porcine vectors with antiserum produced from vaccinated mice demonstrated that these AAVs were distinct serotypes and antigenically different from other more commonly used AAVs. NAbs were absent for each of the porcine serotypes tested even at a concentration of 12 mg/ml of pooled undiluted human sera. However, NAbs were present for the more common AAV2/5 serotype at a concentration of 0.1875 mg/ml of pooled human sera. The absence of neutralizing antibodies to the novel porcine AAV indicates that these serotypes are of low seroprevalence and are not commonly found in humans. Therefore, these porcine AAV serotypes could theoretically be used for therapeutic applications in humans without having to worry about the negative impacts of pre-existing immunity experienced by more commonly-used human AAV vectors.

7.3 – Generation of Porcine H5N1-HA AAV Vectors for Immunological Studies

AAVs have mainly functioned as vehicles for gene therapy due to their nonpathogenic nature and also the apparent lack of host immune responses to these vectors or their encoded transgenes. There are a number of systems that have been proposed to explain the phenomenon associated with AAV vectors such as immunological ignorance, anergy

or deletion and suppression. Despite these factors, a number of groups have more recently shown specific AAV vectors to be efficient at generating robust humoral and cell mediated immune responses in animal models. This has sparked new research into the use of AAV vectors as vehicles for vaccine applications with the hopes of generating protection from infectious diseases [5, 85, 86].

To gain a better understanding and to further characterize the newly found porcine AAVs, immunological studies were carried out *in vivo* with these serotypes. The HA antigen from Hanoi 2005 was chosen as the transgene for the porcine AAV vectors to express in order to measure their immune profiles. Two AAV serotypes AAV8 and AAVrh32.33 that were isolated from rhesus monkeys were used as controls due to their previous history as being either non-immunogenic or immunogenic respectively [5].

Hybrid AAV particles expressing HA were produced by triple transfection and purified by CsCl gradient centrifugation. At the time of production, problems were encountered with the AAV vector preparation yields being of too low a concentration. After troubleshooting many aspects of the production process (e.g. changing CsCl stocks, cell lines, transfection reagents, etc.) a solution was found by slight modification of our existing protocol to include an RT-PCR step (described in section 5.3.6) during fractionation to make viral collection more specific. This modification led to higher yields of AAV vectors during preparations. The purified vectors expressing H5N1-HA were used to infect HEK 293T cells in order to verify proper protein expression and

western blots were performed. Western blots revealed that all of the hybrid vectors were properly expressing the HA transgene and that the protein banding was similar to the positive control.

7.4 – Selection of *In Vivo* Model and Dose for Immune Characterization of AAV

Animal studies were subsequently planned upon verification of proper HA protein expression by the hybrid vectors. For *in vivo* experiments it was decided that BALB/c mice would be used as the animal model for immunological characterization of the novel porcine vectors. BALB/c mice were chosen based on their physiological and immunological similarities to humans in addition to their size, low cost, ease of handling and because they are a lethal animal model for influenza infection. In order to have a successful experiment the proper dose and route of vector administration had to be chosen. The dose and route of administration of AAV vectors also contributes significantly to B and T cell responses. Too low of a vector dose could result in incomplete protection and too high of a dose could result in a dysfunctional immune response or the generation of immune tolerance as described in previous studies [85, 86]. Since our goal was to evaluate host immune responses to the novel porcine vectors we opted to go with an intramuscular (IM) route of administration and a medial dose of 2.5×10^{10} GC/mouse based on comparisons with other groups [5, 86]. All mice vaccinated with 2.5×10^{10} GC of the respective vector remained asymptomatic and had no signs of toxicity to the admistered vectors.

7.5 – *In Vivo* T Cell Responses to Porcine AAVs Expressing HA

The level of cell-mediated immunity following vaccination will determine the potential role of porcine AAV vectors as either gene therapy or vaccine candidates. A strong cell-mediated immune response may circumvent poor humoral responses and would also support the use of vectors for vaccine applications. Since we were dealing with new serotypes that have not been tested it was important to cover a span of time points to get a proper perspective of cell mediated responses. As a result, mice were sacrificed and splenocytes were isolated on days 10, 20, 30 and 40 post vaccination with T cell responses against H5N1 HA peptide pools being detected via secretion of IFN-γ as measured by ELISpots at each time point.

Although most hosts are relatively slow to develop immune responses to AAV, significant responses were detected as early as 10 days post vaccination with the AAV2/8 vector. This was an interesting observation since AAV2/8 is normally seen as non-immunogenic and that one would expect this serotype to have a lower T cell response in comparison to the more immunogenic AAV2/rh32.33 vector, especially at such an early time point. The AAV2/po5 vector consistently had lower T cell responses than any of the other vectors on days 10, 20 and 30 post vaccination but had higher responses than AAV2/po4 and AAV2/8 on day 40. A possible mechanism to explain this trend is that AAV2/po5 poorly transduces murine cells, as was shown in characterization studies involving LacZ and this may cause a proposed delay in cell mediated immunity towards this vector. The immunogenic control vector AAV2/rh32.33 had substantially higher T

cell responses than any of the vectors on days 20 and 30 post vaccination but was surpassed by the porcine AAV2/po6 vector on day 40. T cell responses of the porcine AAV2/po1 and AAV2/po5 vectors were comparable to that of the control vectors at the final day 40 time point whereas AAV2/po6 had the highest response. These results provide valuable insight into early T cell responses that are generated using porcine AAV vectors in comparison to control vectors already in place for gene therapy and vaccine studies. The overall T cell responses generated by the porcine AAV vectors were comparable and in some cases higher than the immunogenic AAV2/rh32.33 control indicating that these vectors are suitable as vaccine candidates.

7.6 – *In Vivo* B Cell Responses to Porcine AAVs Expressing HA

It has been a routine finding that humoral responses to AAV-encoded transgenes are much more intense and more consistently generated than T cell responses [34, 87]. To further our understanding of the porcine AAV serotypes, humoral immune responses were also evaluated in mice through the detection of HI titres following vaccination with AAV. Mice were vaccinated IM with the corresponding AAV vector and 25 days later sera were collected for HI assays. The AAV vectors had similar HI antibody titres and minimal variations were observed between all serotypes tested. All vectors had HI titres above 40 which is the level considered to confer 50% protection in humans as stated by the Centers for Disease Control and Prevention [85].

7.7 – Protection Studies of Porcine AAVs Following Lethal Challenge in Mice

To evaluate the potential use of porcine AAV vectors as vaccine candidates, a lethal challenge in mice with Hanoi 2005 was performed. Mice were vaccinated IM with the consequent AAV vectors and 28 days later were challenged IN with a 100 LD50 dose of Hanoi 2005. All AAV serotypes provided 100% survival with the exception of AAV2/po5 which had a 90% survival rate. All surviving animals did not show any signs of disease and had minimal changes in weight. Mice in both the PBS and AAV2/rh32.33-LacZ control groups did not survive the challenge and the first animals succumbed to disease at day 9 post challenge. Although AAV2/po5 displayed 90% protection, all other porcine vectors gave 100% protection which speaks volumes to their efficacy as vaccine models. Also the fact that mice vaccinated with a non-specific AAV vector did not survive challenge verified that protection was specific to the expression of the HA transgene.

8.0 – Final Thoughts and Future Directions

Our findings demonstrated that porcine AAV vectors are efficient platforms for vaccine applications and that further studies are needed in order to fully understand their role for gene therapy. The low seroprevalence of porcine AAV vectors combined with their ability to transduce murine tissues with similar, or in some cases higher, efficiency than existing vectors is very useful for gene therapy and vaccine applications in humans where issues of pre-existing immunity are of concern. The porcine vectors proved to be effective at providing full protection against a lethal challenge with Hanoi 2005 at 28 days post vaccination. These vectors also generated protective B and T cell responses comparable to those of the immunogenic AAVrh32.33 control, signifying their potential as vaccine platforms.

Additional characterization studies involving the functionality of the host immune response would have added to our understanding of the novel porcine serotypes. Previous work has shown that specific AAV serotypes can trigger functionally aberrant responses leading to anergy and proliferative impairment of $CD8^+$ T-cells [47, 51]. Therefore, it would have been useful to carry out cytotoxic T-cell assays to see if this phenomenon was also observed among the porcine vectors. The presence of non-functional T-cells after administration of specific AAV serotypes *in vivo* would have provided evidence to support the notion that these novel AAV serotypes are also well suited for gene therapy applications in addition to their use as potential vaccine candidates.

In addition to functional immune responses that can identify tolerogenic AAVs, survival experiments based on the AAV vector dose could have also been done and would identify which AAV serotypes are most immunogenic. Such experiments would determine the threshold needed by a specific serotype to initiate an immune response and could identify the best immune stimulators for vaccine applications.

Although we sought out to identify and characterize the porcine AAV serotypes as being either immunogenic or non-immunogenic, our results supported that these vectors were in fact immunogenic under the conditions set forth. While the porcine vectors performed well in transduction studies both *in vitro* and *in vivo*, long term tissue transduction experiments in addition to host immune responses are required to designate these vectors as non-immunogenic. We came to the realization that a number of factors have to be considered when evaluating novel AAV serotypes for use as gene therapy or vaccine vehicles. Factors such as the vector dose, route of administration and capsid structure are all important correlates for the host immune response but these all can be outweighed by the immunogenicity of the transgene being expressed. In our case, the HA antigen that was being expressed by the vectors was highly immunogenic, eliciting a strong host immune response even with the AAV2/8 vector known to be of low immunogenicity. For these reasons, we were only able to effectively designate the porcine AAV serotypes as being immunogenic and our results supported the hypothesis put forward.

Future studies investigating host immune responses specifically directed to the porcine AAV capsid are needed to further elucidate their roles in terms of immunogenicity. A possible experiment to help determine whether the porcine vectors are immunogenic or non-immunogenic would be to vaccinate mice with vectors expressing a non-immunogenic transgene such as a secreted recombinant protein and immunologically recognized as "self." Experiments of this nature have been done in the past using the beta chain of chorionic gonadotropin (β-CG) in NHPs as a marker for gene therapy [25]. This type of experiment would allow for direct comparison of the host immune response to an AAV vector based solely on the vector capsid and not the transgene being expressed.

In conclusion, this study provides valuable insight into the use and characterization of novel AAV serotypes from porcine tissues as candidates for both gene therapy and vaccine applications.

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