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DIVERGENCE, NOT DIVERSITY OF AN ATTENUATED EQUINE LENTIVIRUS VACCINE STRAIN CORRELATES WITH PROTECTION FROM DISEASE

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Abstract

We recently reported an attenuated EIAV vaccine study that directly examined the effect of lentiviral envelope sequence variation on vaccine efficacy. The study [1] demonstrated for the first time the failure of an ancestral vaccine to protect and revealed a significant, inverse, linear relationship between envelope divergence and protection from disease. In the current study we examine in detail the evolution of the attenuated vaccine strain utilized in this previous study. We demonstrate here that the attenuated strain progressively evolved during the six-month pre-challenge period and that the observed protection from disease was significantly associated with divergence from the original vaccine strain.

Keywords

EIAV; Vaccine; Attenuated

1. INTRODUCTION

Attenuated virus vaccines are currently used for the prevention of infectious diseases such as influenza, chicken pox, and yellow fever and have previously been used effectively to control significant viral outbreaks such as smallpox, polio, and measles epidemics [2–4]. However, the use of an attenuated human immunodeficiency virus (HIV) vaccine has been controversial due to concerns that have arisen about vaccine safety [5–10]. Nonetheless, regardless of the potential commercial use of an attenuated vaccine, the model itself is to date one of the best measures of both potential vaccine efficacy and correlates of protection and remains an asset to the field of study. Thus far the development of vaccines to HIV-1 has relied substantially on the use of animal lentivirus models to evaluate the efficacy of various vaccine strategies.

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EIAV, a macrophage-tropic lentivirus, causes a persistent infection in horses and a chronic disseminated disease of worldwide importance in veterinary medicine (reviewed in Craig and Montelaro, 2008 and Montelaro, Ball, et al. 1993). The virus infection, which is transmitted via blood-feeding insects or iatrogenic sources such as contaminated syringe needles, occurs in three stages: acute, chronic, and inapparent. EIA is characterized during its acute and chronic stages by defined episodes of clinical disease triggered by waves of viremia and distinguished by fever, anemia, thrombocytopenia, edema, and various wasting signs. By 8–12 months post-infection horses typically progress to life-long inapparent carriers, but maintain varying steady state levels of viral replication in monocyte-rich tissue reservoirs [11–14]. Stress or immune suppression of EIAV inapparent carriers can induce an increase in viral replication and potentially a recrudescence of disease [15–17]. Among virulent lentiviruses, however, EIAV is unique in that despite aggressive virus replication and associated rapid antigenic variation, greater than 90% of infected animals progress from a chronic disease state to an inapparent carrier stage. This progression to an inapparent stage of disease is achieved by a strict immunologic control over virus replication [13,14]. The EIAV system therefore serves as a uniquely dynamic model for the natural immunologic control of lentiviral replication and disease. Thus, this model provides a novel and useful lentiviral system for identifying critical immune correlates of protection and ascertaining the potential for developing effective prophylactic lentivirus vaccines.

Over the past 20 years we have evaluated a number of experimental EIAV vaccines based on inactivated whole virus, viral or recombinant envelope subunit vaccines, and various attenuated proviruses [1,18–24]. The results of these vaccine trials demonstrate a remarkable breadth of efficacy, ranging from protection from detectable infection and/or disease to severe enhancement of EIAV replication and disease. Amongst our most recent vaccine work are serial studies evaluating the efficacy of an attenuated EIAV proviral vaccine containing a mutation in the viral *S2* accessory gene (EIAV_{D9}) [1,18–20]. The results of these studies indicated that 100% horses inoculated with the EIAV_{D9} viral vaccine were protected from disease by homologous virulent EIAV challenge. Thus, the EIAV system mirrors other animal lentivirus vaccine models that have consistently identified attenuated vaccines, among the various vaccine strategies evaluated to date, as producing the highest level of vaccine protection, typically against homologous virus challenge [25–28].

Our latest published reports of this attenuated vaccine system detail the specific effects of envelope (Env) sequence variation on vaccine protection [1,29–31]. In that study we identified for the first time a significant, inverse, linear correlation between vaccine efficacy and increasing divergence of the challenge virus Env surface gp90 protein compared to the vaccine virus gp90 protein. The vaccine study demonstrated approximately 100% protection of immunized horses from disease after challenge by virus with a homologous gp90, but only 50% protection against challenge by virus with a gp90 that was 13% divergent from the vaccine strain. Immune analysis of potential correlates of protection between the three challenge groups revealed minor associations, but were not definitive. Whether this lack of strong immune system correlations is an accurate statement on *in vivo* immune responses or a call to develop new *in vitro* assays for immune analysis remains to be determined.

A question that has yet to be defined in the field of lentiviral attenuated vaccines is what role potential evolution of the attenuated vaccine, and more specifically, evolution of the vaccines immunogenic target, the Env gene, plays in vaccine efficacy. To date, very little is known about attenuated viral vaccine evolution. Thorough analyses of the attenuated viral replication and interactions with the host immune response, which affect evolution, have been examined, but direct analysis of the actual genomic evolution of the quasispecies population is absent. We know that in order to achieve a protective and yet safe vaccine that the level of attenuation has to be precisely controlled to allow adequate persistent viral

replication and presumably sustained viral antigen presentation necessary to sufficiently drive host immune responses to achieve protective immunity. We have also determined that a second critical parameter for achieving protective immunity with an attenuated vaccine is the length of time post vaccination with the attenuated virus [19,32–36]. However, only a few studies of attenuated lentiviral vaccine evolution have been performed, and the majority of these explored compensatory mutations but not a full quasispecies analysis of population evolution. In this current study we characterize the evolution of the attenuated viral vaccine from our most recent study [1] by examining its viral population at the day of challenge (DOC). Further, we compare it to the longitudinal evolution of its pathogenic viral strain counterpart while also examining its correlation with protective efficacy of the vaccine.

2. MATERIALS AND METHODS

2.1. Experimental subjects, inoculations, clinical evaluation, and sample collection

All equine procedures were conducted at the Gluck Equine Research Center of the University of Kentucky according to protocols approved by the University of Kentucky IACUC. Clinical EIA episodes were determined on the basis of rectal temperature and platelet count in combination with the presence of infectious plasma virus [14,37,38]. CBC analysis of whole blood was performed using an IDEXX QBC Vet Autoreader. Hematocrit and platelet numbers were monitored weekly using the Unopette microcollection system (Becton Dickinson, Rutherford, N.J.). Plasma samples were collected at regular intervals and during each disease cycle (defined as rectal temperature $> 39^{\circ}\text{C}$ and platelet number $< 70,000/\mu\text{l}$ of whole blood) and stored at -80°C until RNA extraction was performed. During the course of these experiments ponies that demonstrated severe disease-associated symptoms resulting in distress as outlined by the University of Kentucky IACUC were euthanized.

2.1.1. Subjects from Attenuated Vaccine Inoculations—Twenty-four outbred Shetland ponies of mixed age and gender and seronegative for EIAV were utilized. Daily rectal temperatures and clinical status were recorded. The EIAV_{D9} attenuated virus stock was produced and vaccinations performed as described [1,18,19]. All animals were vaccinated two times at 30-day intervals by intravenous injection of 10^3 TCID₅₀ as previously described [1]. Vaccinated ponies were challenged six months post-second inoculation with 10^3 TCID₅₀ of EV0, EV6, or EV13 [1]. The ponies were monitored daily for clinical symptoms of EIA.

2.1.2. Subjects from Pathogenic Infections—Two outbred, mixed-breed Shetland ponies were experimentally inoculated intravenously with 10^3 TCID₅₀ of the pathogenic viral strain EIAV_{PV}[23,39,40]. Rectal temperatures and clinical status were recorded daily. The clinical and immune responses of these experimentally infected ponies during acute, chronic, and inapparent infection have been extensively described [12,17,37,41,42].

2.2. Quantitation of Viral RNA, RNA Purification, and RT-PCR

Plasma samples from all animals were analyzed for the levels of viral RNA per milliliter of plasma using a previously described Taqman quantitative real-time multiplex RT-PCR assay based on *gag*-specific amplification primers [43]. The standard RNA curve was linear in the range of 10^1 molecules as a lower limit and 10^8 molecules as an upper limit.

For sequence analysis, viral RNA was extracted from plasma as previously described [16,37,42]. Reverse transcription of 2 to 5 μl of purified viral RNA was performed with the SuperScriptII PreAmplification System (GibcoBRL, Rockville, MD) as specified by the manufacturer using the EIAV specific primer PV12AS [37]. Multiple nested amplifications

of the gp90 Env gene were performed as previously described [42] using the Elongase mix (Gibco BRL, Rockville, MD) and 5µl of the cDNA in a final volume of 50µl.

2.3. Cloning and Sequencing of RT-PCR products

Cloning and sequencing of the EIAV_{PV} pathogenic isolates has been described previously [17,42]. The cloning and sequencing of the DOC attenuated plasma viral populations from all 24 EIAV_{D9} vaccinated animals were performed similarly. Briefly, several independent RT-PCR products (at least 2 independent RT reactions and 2 to 4 independent nested PCR reactions) were generated from plasma samples, gel-purified using Qiagen's Qiaex Gel Purification System (Valencia, CA) and cloned individually into the pCR2.1-TOPO® vector from the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA). Due to the highly unstable nature of EIAV *env* sequences when associated with high copy plasmids, ligations were transformed into Stbl2 cells (Invitrogen, Carlsbad, CA). Clones were screened for the proper size insert by PCR using M13 primers from the TOPO TA Cloning kit. Plasmid DNA was extracted and purified with a midiprep kit (Qiagen, Valencia, CA). Clones (2–5 isolates from each vaccinee) were automatically sequenced with the Taq Dye Deoxy Terminator Cycle Sequencer Kit (Applied Biosystems, Foster City, CA) using internal EIAV primers (variable region 3 through variable region 7) as previously described [37]. DNA sequences were resolved with an ABI Prism 373 DNA sequencer (Applied Biosystems, Foster City, CA). Error rate associated with our nested amplification with the SuperScript and Elongase polymerases was previously determined to be 0.015% (3 substitutions per 19,510 bp sequenced) [37].

2.4. Sequence and Population Analyses

Approximately 200 clones, 75 attenuated EIAV_{D9} DOC Env clones and 120 pathogenic EIAV_{PV} Env clones, were examined for the current study. Sequences were assembled and error checked using Geneious version 4.8.3 (Biomatters Ltd, [44]). Nucleotide and deduced amino acid sequences from each clone were aligned using the Geneious multiple alignment program (a generalization of Needleman-Wunsch) as implemented in Geneious v.4.8.3 and edited manually when necessary [44]. The sequences were aligned using the PAM 250 [45] cost matrix and an open and extend gap penalty cost of 8 and 12 respectively. Divergence/diversity analyses were determined utilizing Jukes Cantor population distance calculations for amino acid sequences with gap weighting set at 0.10 as executed in the program distmat from the EMBOSS package of software. Linear regression and statistical significances of the mean divergence of the pathogenic isolates over time were calculated in Prism version 5.0 (GraphPad Software, San Diego California USA, www.graphpad.com). The statistical significance of the genetic distances in measures of both population divergence and diversity were calculated using two-tailed unpaired t-tests, as implemented in GraphPad InStat version 3.00 (GraphPad Software, San Diego California USA, www.graphpad.com).

In order to evaluate phylogenetics, an alignment of Pony #567 first through six fevers and inapparent populations [1,17] and all of the attenuated EIAV_{D9} DOC populations was generated in Geneious version 4.8.3 [44]. A phylogenetic tree of the hand edited, aligned sequences were constructed by the neighbor-joining method of Jukes Cantor corrected measurements with the optimality criterion set to distance as measured in PAUP [46] and implemented in Geneious version 4.8.3 [44]. Statistical significance of branchings and clustering were assessed by bootstrap re-sampling of 1000 pseudoreplicates on the complete data set; the tree was rooted to EIAV_{PV}. The trees were edited for publication using FigTree version 1.1.2 [47].

3. RESULTS

3.1. Clinical comparisons of vaccination with attenuated EIAV_{D9}, (and subsequent challenge with variant virulent EIAV strains EV0, EV6, and EV13) and experimental infection with pathogenic EIAV_{PV}

3.1.1. Attenuated EIAV_{D9} vaccinations and virulent virus challenge—The vaccinated equids utilized in the present study were part of a large attenuated EIAV vaccine trial that examined the effects of Env sequence variation on vaccine protection. Twenty-four EIAV-naïve ponies were vaccinated two times with 10^3 TCID₅₀ EIAV_{D9} at 30-day intervals and accordingly challenged six months post-second inoculation with one of three virulent EIAV strains EV0, EV6, or EV13 whose gp90 proteins varied from the vaccine Env by 0%, 6%, and 13% divergence, respectively [1]. Challenge results demonstrated approximately 100% protection of immunized ponies from disease after challenge by virulent virus with a homologous gp90, but this level of protection dropped to approximately 63% with a challenge strain whose gp90 was 6% divergent and then further fell to only 50% protection when challenged with a virulent virus containing a gp90 that was 13% divergent from the vaccine strain (Figure 1). Representative clinical and virological profiles of two of the twenty-four ponies, one protected and the other unprotected from disease upon challenge, are presented in Figure 2A and B. There were no remarkable clinical differences between the protected and unprotected ponies during the vaccination period of observation. Large fluctuations in temperature or platelet levels were not observed. Attenuated viral loads displayed minor vacillations, but were at steady state levels typical of our attenuated strain, averaging 10^3 – 10^4 copies/ml viral RNA. The majority of vaccinates had approximately 10^4 copies/ml RNA on the DOC. Lacking any overt or distinguishing clinical or virological (or immunological [29]) relationships with regards to protection from disease, we next assessed the evolution of the attenuated vaccine Env gp90 protein at DOC in this current study. However, before examining attenuated viral gp90 evolution and its association with vaccine efficacy we first sought to fully characterize the evolution by comparing attenuated viral gp90 evolution to pathogenic viral gp90 evolution.

3.1.2. Pathogenic EIAV_{PV} virus infections—The EIAV_{PV}-infected ponies evaluated as part of the current study were part of previous studies from our lab. Throughout these studies we extensively characterized the Env variation that occurred in these long-term inapparent carrier ponies experimentally infected with our pathogenic strain, EIAV_{PV} [17,37,42]. The Env region of EIAV_{PV} is identical to that of EIAV_{D9}. The only viral genomic difference between EIAV_{D9} and EIAV_{PV} is the S2 protein, or site of viral attenuation [20,48,49]. Unlike the clinical profiles of the vaccination period of the attenuated infection, the representative pathogenic profile depicted in Figure 2C illustrates the dynamic and concomitant changes in temperature, platelet counts, and viral loads that occur during pathogenic infection and more specifically during disease episodes. The representative pony in Figure 2C, Pony #567, experienced six febrile episodes, each accompanied by high viral loads (10^6 – 10^9 RNA copies/ml plasma) over the initial observation period. The second pathogenic animal not represented in the figure had a very similar disease course experiencing six febrile episodes during the first year of infection (data not shown). Following their sixth fevers, both ponies entered the inapparent stage of EIA and were asymptomatic throughout the rest of the approximate four year observation period, but maintained low levels of viral replication which averaged approximately 10^3 RNA copies/ml plasma (Figure 2C). The only clear virologic similarity between the attenuated and pathogenic EIAV infections is that the steady-state levels of viral replication are similar during the pre-challenge period for attenuated viral replication and the inapparent stage of disease in pathogenic viral replication. Characterization of Pony #567's quasispecies viral Env sequence (clinical profile Fig. 2C) exhibited elevated levels of gp90 variation and

produced the highest number of emergent viral populations (32 detected populations from inoculum to final inapparent analysis) compared to other experimentally infected ponies in previous analyses [42]. Hence the gp90s for the EV0, EV6, and EV13 challenge strains utilized in the 3.1.1 study were derived from Pony #567 isolates [1].

3.2. Population analysis of EIAV Env gp90 region in pathogenic versus attenuated infections

The current study evaluated the viral sequences of nearly 200 isolated viral clones, 75 clones from the 24 EIAV_{D9}-inoculated ponies and 120 clones from the two EIAV_{PV}-infected ponies. EIAV Env sequences were determined using viral RNA recovered from pelleted plasma virus. The EIAV_{D9} attenuated viral isolates were sampled on the DOC, which was approximately 190 days post-infection (DPI), and the EIAV_{PV} pathogenic isolates were sampled at various febrile and afebrile time points, as indicated in the individual analyses.

For the current study we focused our sequence analysis on the region of greatest variability within the gp90 Env protein, the third variable region (V3) through the seventh variable region (V7). This decision was based on a number of factors. Primarily, the vaccine study that the current attenuated EIAV_{D9}-inoculated ponies came from demonstrated that Env gp90 variation alone dramatically affected protection from disease in vaccinated ponies. Furthermore, the challenge strains EV0, EV6, and EV13 were generated from the Env gp90 regions of EIAV isolates from Pony #567. Finally, previous studies from our lab [17,37,38,42,50,51] and others [52,53] that focused on the genomic variation observed in the gp90 region of the Env gene indicate that EIAV Env variation is localized to specific variable segments of the gp90 surface glycoprotein, namely the V3 through V7 regions.

Since attenuated lentiviral Env population evolution has never been reported, our first goal was to determine whether measurable evolution occurred in the attenuated viral populations. As such, initial characterizations of the attenuated EIAV_{D9} DOC population were carried out through analyses of the divergence of the Env gp90 V3–V7 region from the original inoculum. To demonstrate how this attenuated gp90 evolution compares to wildtype or pathogenic EIAV evolution we evaluated the divergence of both the attenuated and pathogenic isolates together. Divergence was calculated by determining the genetic distance of the deduced amino acid sequences of the viral quasispecies from the original inoculum at the indicated time points. Isolates were obtained from the pathogenic animals during the six febrile episodes (Figure 2C and Figure 3) in each pony and at a single inapparent time point for Pony #567 (from which the EV13 challenge strain Env was derived, Figure 3B). The divergence of the pathogenic isolates from EIAV_{PV} is plotted in Figure 3 as a function of DPI. Both EIAV_{PV}-infected ponies displayed strong, significant ($P < 0.0001$) linear correlations between increasing divergence of the viral strains and time with r^2 values of 0.97 (Pony #564, Fig. 3A) and 0.89 (Pony #567, Fig. 3B). To directly compare these values to the divergence of the attenuated viral isolates two approaches were utilized. The attenuated EIAV isolates that were obtained on the DOC occurred at 190 DPI. Therefore, we initially compared attenuated EIAV_{D9} divergence to the divergence of the pathogenic EIAV_{PV} at sampled time points that occurred closest in their respective timelines to 190 DPI. Hence, we combined the divergence of the population observed for Pony #564 during the fourth fever at 107 DPI (Fig. 3A) and for Pony #567 during the third fever at 230 DPI (Fig. 3B). As demonstrated in Figure 4, there is a higher, statistically significant difference ($P = 0.0093$) in the level of median divergence observed in the pathogenic isolates as compared to the attenuated isolates. The median divergence of the attenuated strains was approximately 1.89% while the divergence of the pathogenic strains was 3.61%. This indicates an approximate 2-fold greater rate of divergence associated with the infection with the pathogenic strain compared to infection with the attenuated EIAV vaccine strain. In our second approach, we chose to calculate a more time-matched, direct comparison of the

divergence of the attenuated and pathogenic virus strains. To do this, we utilized the linearity of the divergence of the longitudinal pathogenic isolates (Fig 3) and the linear regression analyses to calculate the approximate divergence of both Ponies' #564 and #567 viral isolates at 190 DPI, represented in Figure 4 by white dots. The calculated divergence for Pony #564 was 5.4% and for #567 was 4.6%. The divergence values calculated by this second method indicate four to five fold increase in median divergence when comparing directly the specific evolution between an attenuated and pathogenic infection at the exact same time points.

3.3. Evolution of the EIAV_{D9} DOC isolates and protection from disease

3.3.1. Divergence versus Diversity of the Attenuated EIAV_{D9} Isolates—After determining that the attenuated EIAV_{D9} population undergoes substantial evolution in the form of divergence from the inoculating strain, we next sought to evaluate whether this evolution could be associated with protection from disease. To fully characterize the attenuated gp90 V3–V7 population evolution we chose to evaluate both the levels of divergence from the inoculating strain as well as the overall population diversity of the attenuated population at the DOC (Figure 5). While divergence measures how a population has evolved from a previous state or its original state or inoculum, diversity measures the level of variation observed within the population at a single time point. The attenuated viral isolates for these analyses were divided into two subpopulations, protected ponies and unprotected ponies, based upon disease outcome after virulent virus challenge to determine potential associations of divergence or diversity with protection. Diversity was determined by evaluating the intra-subpopulation median genetic distances of the attenuated viral isolates. The median diversity for protected and unprotected ponies was 1.5% and 1.94%, respectively. The median diversity for protected and unprotected animals were similar and surprisingly slightly higher in the unprotected subpopulation. Outliers aside, the range of distances appeared wider in the protected animals, but there was no significant association of diversity between protected and unprotected animals. The median divergence of the attenuated viral isolates from the original EIAV_{D9} of protected and unprotected ponies was 2.67% and 1.89%, respectively. The range of divergence was much greater in the protected animals as compared to the unprotected, median divergence was higher in the protected subpopulation as compared to the unprotected, and was significantly associated with protection ($P = 0.028$). Thus, EIAV vaccine strain divergence provides a statistically significant correlate of vaccine efficacy that is not observed with measurements of viral diversity.

3.3.2. Phylogenetic analysis of the individual isolates from the EIAV_{D9} DOC populations—To elucidate a more detailed picture of how the evolution of the individual attenuated isolates could be related to protection from disease, we performed phylogenetic analyses on the populations from the attenuated and pathogenic variants (Figure 6). A neighbor-joining phylogenetic tree was constructed utilizing the amino acid sequences from the attenuated EIAV_{D9} populations as well as the pathogenic EIAV_{PV} population of Pony #567. The challenge strains EV0, EV6, and EV13 were generated from Pony #567's viral populations (Fig.6). All sequences were aligned with, and rooted to, EIAV_{PV}. All symbols of isolates were color-coded to febrile disease (EIAV_{PV} isolates) or by challenge strain exposure and protection (EIAV_{D9} isolates). As observed previously [17,42], the pathogenic population of Pony #567 increasingly shifts away from EIAV_{PV} with each progressive febrile episode evolving further down the tree from the original EIAV_{PV} root. With minor overlap of populations, each febrile episode in general clusters together, and at the third fever, the populations break away from the main ancestral branch of EIAV_{PV}. The inapparent stage of disease displays the most overlap, which is likely indicative of a lower level viral replication and immune selection. When comparing the attenuated populations to

this pathogenic population, the majority of clones from unprotected animals (open diamonds) fall close to the root of the tree, displaying minor evolution from the original EIAV_{PV} (or EIAV_{D9}) Env gp90 sequence. In fact, 91% of the attenuated isolates from unprotected vaccinates fell on the main root of the phylogenetic tree. Furthermore, the only protected animals with the majority of attenuated isolates located on the main ancestral root were challenged with EV0, which was a homologous Env. Analysis of the isolates from the vaccinated animals that were challenged with the highest divergent strain (EV13) reveals that the main group of isolates from protected animals fall into the branch furthest from the ancestral root of EIAV_{PV}. Finally, all attenuated viral isolates from animals protected from EV13 challenge that fall on the main ancestral root (closer in proximity to EIAV_{PV}) also have a sister clone (from the same animal) that has diverged away onto the next phyletic branch (Fig.6, arrows).

4. DISCUSSION

Among the diverse AIDS vaccine strategies tested to date in animal lentivirus models, attenuated lentivirus vaccines have uniformly provided the highest levels of immunogenicity and protection from disease [20,28,34–36,54–61]. Many important lessons on vaccine immunogen development and correlates of protection can be learned from studies of this highly successful vaccine regimen. Recently, due to the informative nature of attenuated vaccine studies, a Live Attenuated Consortium (LAC) was developed as part of the International AIDS Vaccine Initiative (IAVI) to study and understand the basis for protection as conferred by attenuated vaccines to facilitate the design of improved immunogens [25].

We recently published a report that established a definitive relationship between defined natural lentivirus Env variation and vaccine efficacy. The observed linear relationship between protection from disease and vaccine immunogen divergence predicted a complete lack of protection of immunized horses from disease upon challenge with a virus gp90 that is 23% divergent from the vaccine strain [1]. While this study demonstrated that Env variation was a critical determinant of vaccine efficacy, ultimately there were no obvious immune correlates of protection. The study presented here was designed to evaluate what role, if any, attenuated vaccine strain genomic evolution, specifically Env gp90 gene evolution, played in the protection of these animals.

Evolution of attenuated vaccines is an area of study that has been widely overlooked, especially in the lentivirus field. In general, reports on attenuated viral vaccine evolution are limited. There are a few reports in the HIV/SIV field based on the nef-deleted viral strains [6,62–68]. These studies focus on repair of the deleted gene and/or compensatory mutations that allow the viral attenuation achieved through nef deletion to return to full viral virulence. None of the reports examines genomic evolution at a population level, and they do not investigate Env changes that could affect immunogenicity of the vaccine. A single report of Env sequencing of a SIV attenuated vaccine found very few genomic changes in the attenuated gp120 gene and reported “no significant differences” in the attenuated viral gp120 nucleotide sequence [69]. However, important caveats to this study are that it was performed on isolates from only 2 animals, DNA and not viral RNA was sequenced, and no higher levels of analyses beyond visual inspection of the sequences were performed to characterize the evolution of the gene. Additionally, we found only one previous study on non-lentiviral attenuated vaccine evolution, which examined population evolution of the primary immunogenic target in the attenuated Sabin polio vaccine [70]. This void in the otherwise thorough evaluation of attenuated vaccines could be due to the fact that the majority of commercially approved attenuated vaccines are for viruses that cause acute disease, such as influenza, as opposed to the persistent infection observed with HIV.

Considering that the evolution and associated immunogenic and antigenic variation of virulent lentiviruses is a large obstacle blocking effective vaccine development, it stands to reason that fully understanding how evolution of an attenuated vaccine affects the outcome of long-term protection could provide pertinent information to facilitate better immunogen design.

In the current study we demonstrated that evolution of the attenuated vaccine strain does occur in the six month period of incubation prior to challenge with virulent virus. While the level of evolution as measured by divergence from inoculating strain was significantly lower than what occurs in a pathogenic strain, this presumably reflects the differences in viral replication levels. If allowed to replicate over a much longer period of time, the level of attenuated vaccine strain evolution would likely increase. Hence, this observed divergence of the attenuated vaccine strain could potentially increase the protective efficacy of an attenuated strain over a longer period of time.

Interestingly, the DOC median diversity of the attenuated isolates from protected animals was lower than that observed in unprotected animals, although the difference was not significant. However, the protected animals' viral populations did display a larger range of diversity. In contrast, the levels of divergence from the inoculating strain not only exhibited a larger range compared to the unprotected values but the median gp90 divergence was significantly greater in the protected animals. Therefore, it appears that a higher level of overall evolution (as measured by divergence from the original strain) provided greater protective efficacy than having a quantitatively greater breadth of immunogens present at the time of challenge. Whether the divergence-associated protective efficacy arose from evolution of immunodominant or immunorecessive regions remains to be identified. However, worthy of note is the fact that many current vaccine regimens are considering multiclade vaccines that comprise multiple immunogens in a single presentation. According to our current data, these vaccines might be better presented in the context of multiple or serial presentations to yield "pseudo-evolution" in the absence of an actual evolving immunogen such as an attenuated virus. The current data indicate that presenting immunogens in a context that mimics evolution and higher levels of divergence instead of focusing on multiple antigens at a single time-point or single regimen would be a more successful approach to overcoming lentiviral variation and achieving broader levels of protection.

Phylogenetic analyses presented in Figure 6 further confirmed the concept that evolution in the form of divergence was associated with protection. All of the ponies challenged with the EV13, or the virulent strain 13% divergent from the vaccine strain (in gp90), whose attenuated (pre-challenge) isolates fell only on the root branch of the tree were not protected from disease. In contrast, all animals whose attenuated isolates evolved away from the main ancestral branch were protected from challenge, except for a single EV6-challenged animal; this single exception could be the result of using an outbred population. Regardless, a remarkable observation is the fact that 91% of the attenuated isolates from unprotected animals fell on the main root of the phylogenetic tree.

The observed evolution of the attenuated viral isolates on the DOC could lead to interesting revelations about immune escape and protection from disease. There exists a natural inclination to correlate genomic changes with immune escape, particularly in the case of neutralizing antibodies in the instance of changes in the Env region. To date, we have not been able to correlate protective efficacy of our vaccines with changes in either CTL or neutralizing antibodies [1,18,19,24,29,30,71]. Results from both of these immune analyses have fluctuated and have been inconsistent between different trials. Currently, the main immunological or serological correlation to protection has been a mature antibody response

as defined by the titer, avidity, and conformation analyses of Env-specific IgG responses. These data were discussed in the original paper presenting the vaccine trial data [1]. From our current data we are in the process of trying to dissect better measures of neutralizing antibody protection by mapping out antibody responses from what we have learned of the attenuated strain evolution prior to challenge with the pathogenic EV0, EV6, and EV13.

The data presented in this report demonstrates for the first time that evolution of attenuated vaccine strains does indeed occur and has a significant effect on vaccine efficacy. These studies highlight not only the importance of engineering Env immunogens that elicit a broader and more effective recognition of variant Env species compared to that achieved with an Env immunogen from a single viral isolate, but also bring to light the importance of developing a different presentation system for these immunogens that allows for the evolution of a protective response rather than a single-shot multivalent approach.

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Figure 1. Attenuated viral vaccine induced protection from disease

The percentage of animals within each trial group, EV0, EV6, and EV13 protected from clinical EIA was plotted as a function of days post-infection. Clinical disease is defined as a combination of two-three features such as: rectal temperature above 39°C in conjunction with thrombocytopenia (platelet decrease to $\leq 70,000/\mu\text{l}$ of whole blood), EIAV viral load $\geq 10^5$ as well as other clinical signs of EIA. Fractions at the end of the step graph lines represent the total number of animals protected per group.

Figure 2. Representative clinical and virological profiles of EIAV-infected horses

The profiles depicted in A–C display the different clinical and virological outcomes observed in EIAV_{D9} attenuated viral infections, both (A) protective and (B) nonprotective as opposed to (C) EIAV_{PV} pathogenic viral infections. After inoculation or vaccination of EIAV-naïve ponies with their respective viral strains their rectal temperatures (↓, right Y axis) and platelet counts (---, 1st left Y axis) were followed daily (X-axis). Quantification of the plasma virus loads (◆, 2nd left Y axis) were performed at periodic time points throughout the initial infections, potential fever episodes of the chronic stage and asymptomatic stages as well. Febrile episodes are defined by a achieving a combination of two-three features such as: rectal temperature above 39°C in conjunction with thrombocytopenia (platelet decrease of $\geq 70,000/\mu\text{l}$ of whole blood), EIAV viral load $\geq 10^5$ as well as other clinical signs of EIA. ◆Vax◆, attenuated viral vaccinations characterized in (A, B) occurred via two 30-day I.V. injections of 10^3 TCID₅₀ EIAV_{D9}; ◆Challenge, vaccinated animals depicted in (A, B) were challenged with 10^3 TCID₅₀ I.V. injections of either EV0, EV6, or EV13; +, animal euthanized due to severe disease; ◆Inoculation, pathogenic inoculations represented in (C) consisted of an I.V. injection of 10^3 TCID₅₀ EIAV_{PV}.

Figure 3. Longitudinal divergence of pathogenic EIAV infections

The mean divergence of the EIAV amino acid Env V3–V7 region populations from their inoculum (EIAV_{PV}) in two individually infected equines are plotted in **(A)**, pony #564 and **(B)** pony #567. Each time point represents isolates (approximately 11–24 isolates per assay point, depending on time point) obtained from ponies during febrile episodes of disease except for the 1200 DPI time point in **B**, which is from the inapparent stage of disease. Linear regression (gray line) analysis (GraphPad Prism v. 5.0) in both animals revealed a direct, significant ($P < 0.0001$) linear relationship between time and increasing divergence, with an r^2 value of 0.97 and 0.89 in **A** and **B**, respectively.

Figure 4. Comparison of average pathogenic and attenuated EIAV strain divergence
 Tukey box and whisker plots (GraphPad Prism v. 5.0) of Env gp90 (V3–V7) amino acid divergence from inoculating strain are plotted for both EIAV_{PV} (#564-107 DPI, #567 230 DPI) and EIAV_{D9} (DOC, 190 DPI) viral populations. For direct comparison to the EIAV_{D9} time-point, calculated or predicted divergence of #564 and #567 190 DPI populations from linear plots in Figure 3 are overlaid in white dots (□). *, Statistical significance of the differences between the two populations (box and whisker plots) at each time point was performed using an Unpaired two-tailed t-test with Welch's corrections. The differences in the medians ($P = 0.0093$) are statistically significant. Outliers in the population are represented by (●) black dots. DPI, days post-infection; DOC, day of challenge.

Figure 5. Average DOC levels of diversity and divergence of attenuated EIAV vaccine strain Tukey box and whisker plots (GraphPad Prism v. 5.0) of the attenuated viral populations Env gp90 (V3–V7) **(A)** diversity and **(B)** divergence from the DOC are graphed as a function of vaccine protection (approximately 34 isolates from unprotected animals and 41 isolates from protected animals). **(A)** The differences in the variances ($P < 0.0001$) are statistically significant. **(B)** The differences in the median ($P = 0.028$) and variances ($P = 0.0016$) are statistically significant. *, Statistical significance of the differences of the two populations was performed using Unpaired two tailed t-tests with Welch's correction. Outliers in the population are represented by (●) black dots. DOC, day of challenge.

Figure 6. Phylogenetic analysis of attenuated EIAV vaccine strain

The phylogenetic relationship between the viral populations of the attenuated virus at DOC and the viral populations of the pathogenic virus from longitudinal time-points (from which the vaccine challenge strains were generated, Pony #567) were examined to elucidate clone specific information. A neighbor-joining tree of aligned Env (V3–V7) amino acid sequences was constructed from Jukes Cantor corrected distances with the optimality criterion set to distance. The tree was rooted to EIAV_{PV} Env (identical to EIAV_{D9} Env). Bootstrap values were determined over 1000 iterations and are indicated at the nodes of the branches. Branch lengths are proportional to the distance existing between the sequences. Color-coded arrows indicate isolates from the same vaccinee. DOC populations: closed diamond = protected from clinical disease, open diamond = unprotected from clinical disease.