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BRIEF REPORT

In vivo evolution of the gp90 gene and consistently low plasma viral load during transient immune suppression demonstrate the safety of an attenuated equine infectious anemia virus vaccine

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Abstract To study the in vivo evolution of the attenuated Chinese equine infectious anemia virus (EIAV) vaccine, viral *gp90* gene variation and virus replication in immunosuppressed hosts were investigated. The results showed that after vaccination, the *gp90* gene followed an evolutionary trend of declining diversity. The trend coincided with the maturation of immunity to EIAV, and eventually, the *gp90* gene became highly homologous. The sequences of these predominant quasispecies were consistently detected up to 18 months after vaccination. Furthermore, after transient immune suppression with dexamethasone, the plasma viral RNA copy number of the vaccine strain in three vaccinated ponies remained consistently below the "pathogenic threshold" level, while the viral load increased by 25,000-fold in the positive control of an inapparent carrier of the parental virulent strain. This study is the first to provide evidence for the safety of an attenuated lentiviral vaccine with decreased genomic diversity and consistently low viral replication under suppressed immunity.

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Lentivirus-induced diseases pose serious threats to both humans and other animals. Human immunodeficiency virus-1 (HIV-1), a lentivirus that causes acquired immunodeficiency syndrome (AIDS), is responsible for more than 5 million new infections annually and has resulted in more than 20 million deaths worldwide [3]. Equine infectious anemia virus (EIAV) is another member of the family *Retroviridae*, genus *Lentivirus*, that infects equines. This virus is present in many countries and causes significant economic losses, especially in developing countries such as China (Feng JL and Shen RX, personal communication and [13]).

Development of effective vaccines against lentiviruses remains a major challenge. In the process of HIV-1 vaccine development, researchers have employed a variety of strategies, including subunit vaccines, live vector-based envelope protein-expressing recombinant vaccines, and DNA prime/protein boost protocols as well as genetically engineered live attenuated simian immunodeficiency virus (SIV) and simian–human immunodeficiency virus (SHIV) vaccines (reviewed in refs. [14] and [22]). Among the existing experimental vaccines, only live vaccines have been found to induce comparatively strong resistance to infection by SIV/SHIV in non-human primates [1, 9, 35, 36]. Other experimental vaccines induce certain levels of immune responses but do not provide sterile protection against infections by pathogenic strains [19].

Safety has been the major obstacle for developing live attenuated lentiviral vaccines, especially for HIV-1. Major concerns are the possibility of live virus vaccines having increased pathogenicity in individuals with dysfunctional immune systems and reversion to virulence during their long-term replication in hosts. Such events actually occurred in studies of attenuated SIV and SHIV vaccines, developed by deletion or mutation of the *nef* gene [11, 20,

29, 33]. While these safety issues have greatly limited research on live HIV-1 vaccines for humans, there has been a successful application of an attenuated lentiviral vaccine for horses. In the 1970s, a Chinese attenuated EIAV vaccine developed by Shen et al. [24, 27] successfully controlled the prevalence of equine infectious anemia (EIA) in China. During a 15-year period, over 70 million equines were safely immunized, and the safety of the vaccine was fundamentally established (Feng JL and Shen RX, personal communication). In addition, in vivo passages and vertical transmission tests of the vaccine also showed no reversion of virulence (Feng JL and Shen RX, personal communication and [23]). Our recent work on the Chinese attenuated EIAV vaccine, including characterization of the in vivo evolution of the *gp90* gene and the interaction between the vaccine strain and its hosts under immunosuppression, further suggest that this attenuated EIAV vaccine is safe for practical immunizations.

Some published studies showed that particular sequences of the gp90 protein, i.e. the surface subunit of the EIAV envelope protein (SU), were closely related to the immunogenicity and virulence of viruses [2, 12]. In order to understand the replication and evolutionary features of the attenuated Chinese $EIAV_{FDDV}$ vaccine in vivo, we analyzed the *gp90* gene of the virus from immunized hosts at different time points by reverse transcriptase-polymerase chain reaction (RT-PCR) and nucleotide sequencing. The analysis of virus diversity based on the *gp90* gene was performed by random selection of multiple clones of PCR products for sequencing. Reverse transcription was performed with the primer p8, 5′-TGACCCCATGATT CATTCCA-3′ (nucleotide positions 6980–6999 of the genome of a Chinese attenuated EIAV vaccine strain, GenBank accession no. AF327878) and a reverse transcription kit (Invitrogen). Subsequently, PCR was performed with primers p7, 5′-CCACCAGAGTGTTGTG GAAAGGTG-3′ (5022–5045) and p8 to amplify a fragment of approximately 1.9 kb. Because the copy number of plasma genomic RNA of $EIAV_{FDDV}$ was low, highly sensitive nested PCR was utilized to amplify the *gp90* gene from samples that were pre-ultracentrifuged to concentrate viral particles. The process of nested PCR for amplifying the $EIAV_{FDDV}$ *gp90* gene was similar to that for reverse transcription. Nested PCR primers included the outer primers p7 and p8 and the inner primers p7-1, 5′-TT GTAAGGTTTGGTGTATGGG-3′ (5282–5302) and p8-1, 5′-ATGGCAGCTATTATAGCAGA-3′ (6680–6699). This fragment obtained by the nested PCR was about 1.41 kb, which was large enough to encompass the entire *gp90* coding region.

DNA polymerase *ExTaq* (Takara) was used for PCR amplification. This DNA polymerase synthesizes DNA with higher fidelity than the common *Taq* DNA polymerase and results in fewer errors in amplified fragments. The amplified gene fragments were then inserted into the pMD18-T vector and introduced into *E. coli* DH5α competent cells by transformation. Twelve to fifteen positive clones were picked for each PCR product and sequenced. DNA Star, Mega 4.0 and GENEDOC were used to analyze sequencing data. Proper negative controls and a positive control of a molecular clone of $EIAV_{FDDV3\text{-}8}$ *gp90* were added to RT-PCR and nested PCR. Fifteen clones from $EIAV_{FDDV3\text{-}8}$ *gp90* were also sequenced to rule out potential contamination (data not shown).

Phylogenetic analysis of PCR clones of the $EIAV_{FDDV}$ *gp90* gene showed that sequence diversity among these clones was high (Fig. 1). The nucleotide variations among the different clones reached as high as 4.0%, and differences in deduced amino acids were up to 6.5% (data not shown). The high diversity among these *gp90* gene clones is considered to be due to the in vitro attenuating process of the parental virulent strains in cells cultured for more than 100 generations. In the process, mutations in viral genomes were accumulated during replication of each generation due to the lack of immunological selective pressure in the cultivation environment.

Phylogenetic analysis on the *gp90* gene of the attenuated vaccine in vaccinated ponies was also performed. The *gp90* sequences at the time point of 15 days post-infection (DPI-15) were closely related to the *gp90* population of the original in vitro-prepared $EIAV_{FDDV}$ in their genotypes and diversity (data not shown). However, the diversity among the *gp90* clones at each time point of in vivo evolution of the vaccine strain was largely different (Fig. 1a). The diversity of the *gp90* gene sequences from the four experimental ponies followed a declining trend for up to 18 months after vaccination, gradually decreasing from $1.45 \pm 0.51\%$ in the early stage after immunization (DPI-15) to $0.51 \pm 0.09\%$ and $0.29 \pm 0.04\%$ in the mature immune stages (DPI-180 and DPI-540, respectively). The values of *gp90* gene diversity were calculated using Dnasp4.0 software.

To illustrate the genetic relationships between the vaccine strains evolving in vivo over time after vaccination, a phylogenetic tree for determining the genotypes of all of the *gp90* sequences, shown in Fig. 1a, was made using Mega 4.0 (Fig. 1b). The distribution of each analyzed sequence in Fig. 1a, b suggested that (1) *gp90* evolved over the time of infection, especially during the earlier stages of infection (before DPI-180), and (2) the diversity of *gp90* had shifted from highly heterologous at time points DPI-15 and DPI-60 to homologous at DPI-180 and DPI-540. These results indicate a tendency of self-limitation of diversity of the EIAV vaccine strain $EIAV_{FDDV}$ in vivo accompanying the maturation of the immunity.

Comparison of the deduced SU amino acid sequences from a total of 52 clones of the vaccine strain $EIAV_{FDDV}$

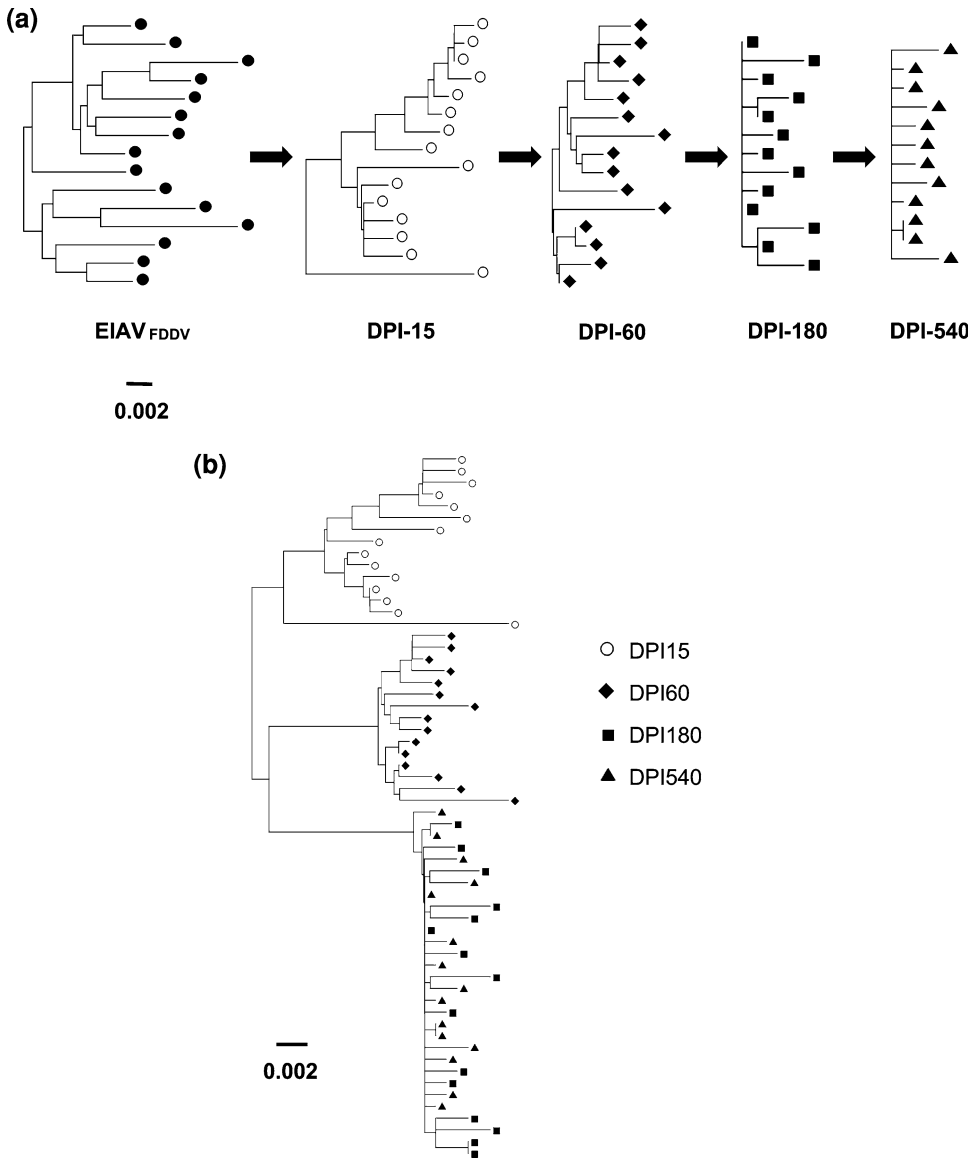

Fig. 1 Phylogenetic analysis of $EIAV_{FDDV}$ *gp90* gene evolution in vaccinated ponies. $EIAV_{FDDV}$ *gp90* gene evolution in one of the four vaccinated ponies is presented. Similar evolution patterns were detected for the other experimental ponies (data not shown). **a** Changes of *gp90* diversity in vivo during the experimental period. Twelve to fifteen clones were sequenced at each time points. **b** Sequences of all of the *gp90* clones shown in **a** were used for phylogentic analysis of in vivo evolution over the experimental period. The *bar* in figures represents 0.002 amino acid changes per site. *DPI* day post-injection

and the relevant sequence of the parental virulent strain $EIAV_{Liao}$ indicated that there were 11 consensus mutation sites in all of the sequences of the vaccine clones (Table 1). Two of these 11 consensus mutations, S193 → N and N237 → K, removed two potential N-linked glycosylation sites in SU. Some studies have shown that removal of the N-linked glycosylation sites in the envelope protein of HIV-1 or SIV enhances the neutralizing antibody response [17, 21]. Therefore, the deletion of these two N-glycosylation sites of pathogenic EIAV SU is presumed to elicit higher levels of neutralizing antibody. It was reported that the reverse mutation of four amino acid residues in SU of an attenuated EIAV strain to the residues of its parental virulent strain resulted in an increased replication rate in vivo and a reversion of virulence [27]. These published studies suggest that the 11 consensus mutations in the $EIAV_{FDDV}$ SU are at least partially involved in the attenuated virulent and enhanced immunogenicity of the Chinese EIAV vaccine strain.

Interestingly, 5 of the 11 consensus mutation sites had reverse-mutated into the corresponding residues of the parental virulent strain in some clones at DPI-60, i.e. residues E189 → K, K190 → E, K237 → N, Q410 → H, and an insertion of 236D (Table 1). However, the other parts of SU sequences retained the characteristics of the vaccine strain. At DPI-60, the plasma viral loads of the four ponies were 10^3–10^5 copies/ml, which was within normal levels for EIAV vaccine strains. In addition, no clinical signs of EIA were observed. To some extent, these results suggested that the partial reverse mutations at these amino acid residues did not alter the attenuated virulence of the vaccine strain $EIAV_{FDDV}$. However, inoculation of these reverse-mutated clones into naïve animals is required to confirm that they are indeed completely avirulent.

Table 1 Comparison of consensus mutation sites in deduced SU amino acid sequence of the vaccine strain $EIAV_{FDDV}$ and its derived viruses sequenced from plasma in vaccinated ponies at different time points

EIAV strains and in vivo species	Consensus mutation sites in SU of the vaccine strain											Divergence from $EIAV_{Liao}$ (%)
	46	98	100	103	189	190	193	236	237	321	410	
$EIAV_{Liao}$	A	G	K(H)	H	K	E	S	D	N	K	H	–
$EIAV_{FDDV}$	E	R	Q	N(Y/C)	E	K	N	–	K	E(N)	Q	5.78
DPI-60[a]	E	R	Q	Y(N)	**E(K)** **6/60**	**K(E)** **3/60**	N	**D** **15/60**	**K(N)**[b] **30/60**	E(N)	**Q(H)** **15/60**	4.60
DPI-180	**E(A)** **15/54**	R	Q	Y	E	K	N	–	K	E	Q	5.26
DPI-540	E	R	Q	Y	E	K	N	–	K	E	Q	5.36

[a] DPI-60, DPI-180 and DPI-540 designate 60, 180 and 540 days post-immunization, respectively. Twelve to fifteen clones were randomly picked from each of the four experimental ponies. *Underlined* and *bold* amino acid residues indicate reverse-mutated sites, and the numbers under these sites are rates of mutation in total analyzed clones. Divergences between the virulent strain $EIAV_{Liao}$ (refers to the Liaoning strain of EIAV, GenBank No: AF327877) and the in vivo species of the vaccine strain $EIAV_{FDDV}$ are shown as the average of analyzed clones

[b] The reverse mutation from K → N at position 237 resulted in the recovery of an N-linked glycosylation site

In addition, reverse mutations of these five residues were not identified in most *gp90* clones at DPI-180 and DPI-540, except one for reverse mutation that appeared at another consensus vaccine-specific site (E46 → A) in clones from one of the four ponies at DPI-180 (Table 1). At this time point, the predominant quasispecies of $EIAV_{FDDV}$ were formed and became stable in the vaccinated ponies. The percent identity of the *gp90* sequences of DPI-180 clones to the corresponding sequences of some predominant $EIAV_{FDDV}$ clones was approximately 99% (data not shown), and the 10 specific consensus amino acid mutation sites of the vaccine strains were fully preserved. Furthermore, the reverse mutation E46 → A no longer existed in the *gp90* clones of $EIAV_{FDDV}$ isolated at the later time point (DPI-540). Such highly homologous and $EIAV_{FDDV}$ reference-strain-like quasispecies were predominant even 18 months after the immunization (DPI-540). Our finding of certain highly homologous $EIAV_{FDDV}$ quasispecies coexisting with other quasispecies in the host during the early stage post-immunization was different from the finding of Craigo et al. [7]. Their study showed that the ancestral species in a long-term inapparent EIAV carrier were eliminated and were substituted by new populations in response to ongoing immune pressure.

Although the attenuated vaccine strain developed reverse mutations at certain amino acid residues in the SU at the DPI-60 time point, the above results indicated that these mutated viral species could be substituted by species with SU characteristics of the attenuated vaccine strain after the maturation of specific immunity (6 months after immunization, DPI-180). These vaccine species continuously existed in the vaccinated hosts for up to 18 months in this study. The exact mechanism for the transient reverse mutation is not clear. However, the combination of the genomic stability and the reduction in diversity of the vaccine strain in the hosts with matured immunity indicates that the Chinese attenuated EIAV vaccine strain had stabilized in vivo, which is an extremely important requirement for a safe attenuated vaccine.

These data indicate that even though the attenuated vaccine strain with variant *gp90* sequences could retain their diversity at the early stage of infection, the genomic variation was obviously reduced with the maturation of protective immunity. Genetic variation could increase the rate of reverse mutations of the attenuated vaccine and therefore increase the danger of virulence reversion. However, the evolutionary trend, from diverse to homologous, of the Chinese EIAV vaccine strain in vaccinated ponies implied that vaccine-induced immunity may play a major role in restricting the diversity of the vaccine strain and driving the genotypes to singularity. If this is true, such immune-driven constraints on the virus would help in reducing the chance of virulence reversion of the attenuated vaccine during replication. Additionally, factors other than pressure from the immune system may have contributed to the observed reduction in SU complexity in vivo, such as selection pressure of re-adaptation from donkey cells to horse cells and from in vitro to in vivo [4].

The major reason for the rare acceptance of attenuated lentiviral vaccines is the risk of increased virulence due to viral mutations when viruses replicate in the host for a long period of time. This concern is especially prominent in the context of decreased immunity, which may result in a dramatic increase of viral load and result in enhancement of pathogenicity of attenuated vaccines [34]. In this study, the immune systems of the vaccinated ponies were transiently suppressed by two weeks of daily administration of dexamethasone. The replication levels and virulence of the vaccine strain $EIAV_{FDDV}$ were investigated by monitoring the change in plasma viral load and appearance of EIA

clinical symptoms. Three ponies, #7, #8 and #9, which had been vaccinated with $EIAV_{FDDV}$ for 18 months, were used to study the change in virulence of the vaccine strain in immune-suppressed hosts. An inapparent EIAV carrier (#6), which was confirmed EIAV positive by sequencing of the *gp90* gene and agar gel immunodiffusion test for the antibody, was used as a positive control. Rectal temperatures were monitored daily from 2 days before administration of dexamethasone until 6 months after the drug treatment. Blood cell count and classification, platelet count and plasma viral load were also regularly measured.

Immunosuppression was induced in ponies in this experiment by a procedure described previously [5, 31]. Dexamethasone was injected intramuscularly at 0.11 mg/kg of the body weight for each pony for 14 days. The effects of immunosuppression were evaluated using a delayed-type hypersensitivity (DTH) test [5] by intradermal injection of 50 μg/ml PHA-P (Sigma) diluted with saline. In addition, lymphocyte proliferation [30] in peripheral blood mononuclear cells (PBMCs) in response to PHA-P stimulation was monitored before and after treatment with dexamethasone. Results of the DTH test and lymphocyte proliferation indicated that the immunity of all the four ponies was significantly suppressed (Fig. 2a, b).

Plasma EIAV loads in the ponies before and after immunosuppression were measured by real-time RT-PCR. The results are shown in Fig. 2c. The viral copy number of the EIAV virulent strain carrier (pony #6) increased 25,000-fold in 10 days after administrating the drug, reaching 2.5×10^8 copies/ml, which was higher than the "pathogenic threshold" of 10^7 RNA copies/ml for clinical EIA. The increased EIAV plasma load corresponded to the appearance of other EIA clinical signs, such as fever and thrombocytopenia (data not shown). As a result, it can be determined that the unapparent EIAV carrier, pony #6, developed an acute episode of EIA when the immunity was suppressed. These EIA symptoms disappeared when the dexamethasone treatment ended. These results indicated that clinical EIA may be induced in long-term asymptomatic carriers of EIAV pathogenic strains when the immunity of the host was suppressed, due to the dramatic increase in plasma viral load.

In contrast, the viral loads of the immunized ponies, #7 and #9, did not show any EIA symptoms during the drug treatment and thereafter. The viral loads of $EIAV_{FDDV}$ were between 10^3 and 10^5 copies/ml. The viral load in pony #8 increased slightly 10 days after the start of dexamethasone treatment and 14 days after the end of drug treatment, but at both times it remained below 10^6 copies/ml, which was within the dynamic levels for the vaccine strain $EIAV_{FDDV}$. Neither the vaccinated ponies nor the inapparent carrier showed any EIA clinical signs during the following 6 months of observation.

Viral load monitoring in these experiments revealed two important facts: First, the immunity, especially the T cell-mediated immunity, induced by attenuated EIAV vaccine

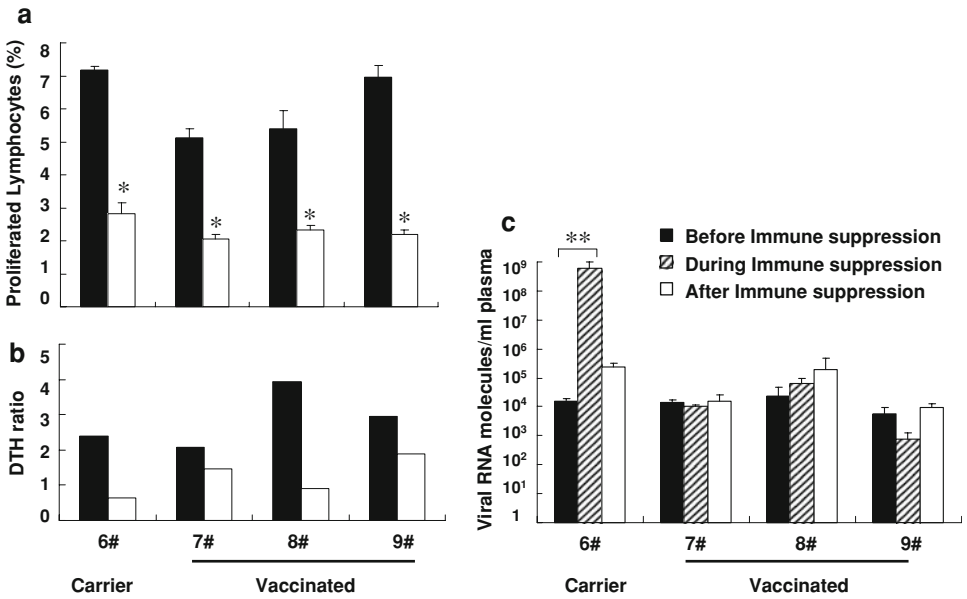

Fig. 2 Evaluation of the immunosuppressive effects and plasma viral loads before and after dexamethasone treatment. $EIAV_{FDDV}$-vaccinated ponies (#7, #8 and #9) and an inapparent EIAVLiao carrier (#6) were injected intramuscularly with dexamethasone every day for 2 weeks. The proliferation of lymphocytes stimulated with PHA-P (**a**) was measured, and a delayed-type hypersensitivity test (DTH, **b**) was performed to evaluate the efficiency of immunosuppression. EIAV RNA copy numbers were measured 2 days before dexamethasone treatment, 10 days after the start of treatment, and 14 days after the end of treatment (**c**). $^*P < 0.5$, $^{**}P < 0.01$ as analyzed using SAS 8.1

was not the major reason for the maintenance of a consistently low level of replication of the vaccine strain in vivo. This low-level replication may be different from that of the virulent strain in inapparent carriers, which was apparently restricted by the immune pressure. This consistently low-level replication of $EIAV_{FDDV}$ in immune-suppressed hosts strongly demonstrated the safety of the attenuated vaccines. Secondly, no clinical EIA symptoms were observed in the vaccinated ponies within at least 6 months after the end of immune suppression. This fact indicated that replication of the attenuated EIAV vaccine in immunosuppressed hosts did not cause any visible health problems, which provided further evidence for the safety of $EIAV_{FDDV}$.

The development of the Chinese attenuated EIAV vaccine was obviously different from that of $EIAV_{D9}$, a live EIAV vaccine that was developed by Li et al. [15] and Craigo et al. [6] by introducing mutations into a defunct *S2* gene. This vaccine strain completely protected immunized horses from infection with a parental virulent strain virus with homologous SU sequence. However, the effectiveness of $EIAV_{D9}$ in protecting animals from infection and disease was significantly reduced when the immunized animals were challenged with viruses containing a relatively modest variation in SU [8]. Since the life cycle of attenuated vaccine virus in the body is far closer to the infected pathogenic lentiviruses than any other types of vaccine, this study provides valuable information for future studies on animal lentivirus and human AIDS vaccines.

The Chinese attenuated EIAV vaccine strain $EIAV_{FDDV}$ was developed by consistently passing the parental virulent strain $EIAV_{Liao}$ in cultured donkey monocyte-derived macrophages and fetal donkey dermal cells for 125 and 15 generations, respectively [25]. This long-term in vitro attenuation process resulted in high rates of mutation in multiple genes of the vaccine strain, mainly LTR, *env* and accessory genes *S2* and *rev* [32]. Several published studies have demonstrated that mutations in these genes altered the pathogenicity of wild-type strains [10, 16, 18, 28, 37].

Accompanied by the variation of genotypes, the phenotype of $EIAV_{FDDV}$ is different from that of $EIAV_{D9}$, including the effectiveness of its immune protection against heterogeneous virulent strains. Previous results showed that $EIAV_{FDDV}$ had successfully protected more than 70% of vaccinated animals from disease after infection by the heterogeneous virulent strain $EIAV_{Wyoming}$ [26].

The attenuation resulting from multiple-site mutation is the major difference between the attenuated Chinese EIAV vaccine and most of the other attenuated lentiviral vaccines, which were developed by recombinant techniques. The development of attenuated SIV or SHIV vaccines has been based primarily on the deficiency or mutation of a single gene (such as *nef*). Because the number of modified sites was limited, and because they were located only in certain segments of the viral genome, the possibility of virulence reversion was high during long-term replication in vivo, as observed in some studies in non-human primates [11, 20, 29, 33, 34]. Since the Chinese EIAV vaccine strain $EIAV_{FDDV}$ was attenuated through multiple-gene and multiple-site mutations, it is logical to assume that the possibility of virulence reversion is substantially reduced. In addition, the unique in vivo evolution pattern and stable, moderate-level replication feature of the vaccine strain is considered favorable for the induction of protective immunity, which could further reduce the risk of virulence reversion of attenuated EIAV vaccines.

In conclusion, our data reveal that the in vivo evolution of the *gp90* gene from $EIAV_{FDDV}$ shows an evolutionary trend going from polymorphism to singularity. There were also some occurrences of reverse mutations in the vaccine strain in hosts before the maturation of immunity. However, such reverse mutations were limited in number and were eliminated with the maturation of immunity. Combined with the consistently moderate level of replication, even in immunosupressed hosts, the Chinese attenuated EIAV vaccine strain appears to be a unique attenuated lentiviral vaccine. Further studies are required to compile the safety profile of this vaccine and to elucidate the mechanisms of attenuated virulence and enhanced immunogenicity.

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Conflict of interest statement The authors declare that they have no conflict of interest.

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