



# Assessing the efficacy of a live vaccine against avian encephalomyelitis virus

Wencheng Lin<sup>1,2,3,4</sup> · Piaopiao Lu<sup>1</sup> · Aijun Li<sup>5</sup> · Yu Wu<sup>1</sup> · Hongxin Li<sup>1</sup> · Feng Chen<sup>1,2,3,4</sup> · Jingyun Ma<sup>1,2,3,4</sup> · Qingmei Xie<sup>1,2,3,4</sup>

Received: 8 February 2018 / Accepted: 19 April 2018 / Published online: 16 May 2018  
© Springer-Verlag GmbH Austria, part of Springer Nature 2018

## Abstract

Avian encephalomyelitis virus (AEV) causes typical neurological symptoms in young chicks and a transient drop in egg production and hatchability in adult laying birds, resulting in huge economic losses in the poultry industry. An effective way to control and prevent this disease is vaccination of the flocks. Here, we assessed the efficacy of the live vaccine candidate strain GDt29 against avian encephalomyelitis virus. The GDt29 strain has low virulence, was confirmed safe, and showed no signs of pathogenicity. High titers of AEV-specific antibodies were detected in GDt29-vaccinated hens ( $S/P > 3.0$ ) and their progeny ( $S/P > 2.0$ ). Moreover, the eggs of GDt29-vaccinated hens with high levels of maternal antibodies were hatched successfully regardless of challenge with a heterologous AEV strain, and the GDt29 attenuated vaccine showed higher protective efficacy against AEV than the commercial vaccine. Furthermore, contact-exposed chicks bred with GDt29-vaccinated birds generated high titers against AE virus ( $S/P > 2.8$ ). Collectively, our studies are proof of the principle that GDt29 might be an ideal vaccine candidate to prevent AEV infection, and they highlight the utility of using a live vaccine against AEV.

## Introduction

Avian encephalomyelitis (AE), caused by AE virus (AEV), is a neurotropic disease that occurs in chickens, pheasants, turkeys and quail, causing considerable economic losses in the poultry industry [1, 2]. AEV can be transmitted vertically

or horizontally and causes neurological symptoms characterized by ataxia, paresis or paralysis in young chickens. It also causes a transient drop in egg production and hatchability in adult egg layers [3–6]. AE is considered an important and ubiquitous disease of birds.

AEV belongs to the genus *Tremovirus* of the family *Picornaviridae* and has a positive single-stranded genome with a large open reading frame (ORF). Like other picornaviruses, the ORF codes for a polyprotein that comprises both non-structural and structural elements divided into three primary precursor molecules — P1, P2 and P3 — encoding 11 distinct proteins, including four structural proteins (VP4, VP2, VP3 and VP1) in the P1 region and seven nonstructural proteins in the P2 and P3 regions [7, 8]. The function of the AEV proteins have often been assigned by virtue of their similarity to their well-studied counterparts in poliovirus (PV), Theiler's murine encephalomyelitis virus (TMEV), and foot-and-mouth disease virus (FMDV).

To date, no treatment or medicine for AEV is available, and the only effective way to control and prevent this disease is vaccination of flocks [9]. Breeder flock vaccination programs designed to provide progeny with maternal antibodies can result in better performance of progeny and prevent transovarian transmission during the period of greatest susceptibility 1–3 weeks after hatching [10]. In China,

---

Handling Editor: Zhenhai Chen.

---

Wencheng Lin and Piaopiao Lu are contributed equally.

---

✉ Qingmei Xie  
qmx@scau.edu.cn

<sup>1</sup> College of Animal Science, South China Agricultural University, Guangzhou 510642, People's Republic of China

<sup>2</sup> Guangdong Provincial Key Lab of Agro-Animal Genomics and Molecular Breeding, Guangzhou 510642, People's Republic of China

<sup>3</sup> Key Laboratory of Animal Health Aquaculture and Environmental Control, Guangdong 510642, Guangzhou, People's Republic of China

<sup>4</sup> South China Collaborative Innovation Center for Poultry Disease Control and Product Safety, Guangzhou 510642, People's Republic of China

<sup>5</sup> College of Science and Engineering, Jinan University, Guangzhou 510632, People's Republic of China

vaccination is mainly done in 14- to 16-week-old breeders by administering a live field virus by the natural route of infection in their drinking water, or by wing-web inoculation through intracutaneous injection [10–13]. This immunity not only protects hens during laying but also protects their progeny through maternal antibodies [4, 14]. The vaccine against AEV that is widely used in the Chinese poultry industry is an inactivate vaccine. This vaccine frequently induces low antibody titers against AEV in breeder flocks, causing them to remain susceptible to AEV during or prior to the onset of production and to transmit AEV to their progeny.

In the present study, we developed a live vaccine against AEV that exhibited a fine balance between attenuation and immunogenicity. The live vaccine should be considered as a candidate vaccine to prevent infection by AEV.

## Materials and methods

### Ethics statement

The animal experiments were approved by the Animal Care Committee of the College of Animal Science, South China Agricultural University, Guangzhou, China (approval ID: 201004152). All study procedures and animal care activities were conducted in accordance with the national and institutional guidelines for the care and use of laboratory animals. The birds were maintained in isolators with negative pressure, and food and water were provided *ad libitum*.

### Viruses and animals

A commercial freeze-dried live vaccine (ENCEFAL-VAC, Calnek-1143 strain, batch no. 506678) was purchased from FATRO S.P.A Ozzano Emilia Bologna, Italy. The challenge strain Van Reokel (AY517471, standard highly pathogenic strain) was purchased from ATCC (ATCC no. VR-713). Fertilized White Leghorn specific-pathogen-free (SPF) eggs and SPF chicks were purchased from the Guangdong DHN Poultry and Egg Products Co. Ltd., China.

### Virus isolation

In 2015, an epidemiological survey was performed on breeder chicken farms to investigate the AEV epidemic overall. A total of 667 clinical samples including brain, pancreas, intestine, myeloid and heart were collected from unvaccinated birds from six commercial chicken farms. Among these 667 specimens, 100 specimens (including four samples collected from diseased 6-day-old birds that exhibited neurological symptoms characterized by ataxia and paresis) were collected from a commercial chicken farm in Jiangxi province, 143 samples were collected from a commercial

chicken farm in Hunan province, and 424 specimens were collected from four chicken farms in Guangdong province.

Viral genomic RNA was extracted separately from homogenized tissues and subjected to RT-PCR for the detection of AEV. Virus isolation and purification were performed using SPF chicken embryos as described previously [15]. Briefly, a pathogenic organ homogenate suspension in PBS containing penicillin (100 U/mL) and streptomycin (100 µg/mL) was filtered for sterilization using a 0.22-µm filter and used as inoculum for AEV isolation. The inoculum was inoculated into a 6-day-old SPF chick embryo by the yolk sac route, and the brain and internal organs were harvested at 10 days postinfection (dpi). The homogenate suspension of the brain and internal organs was prepared in PBS, frozen and thawed three times, and then clarified by centrifugation. AEV was purified by sucrose density gradient centrifugation. The AEV isolates were serially passaged up to 15 times by inoculating 6-day-old fertilized SPF eggs by the yolk sac route. Virus was detected using an RT-PCR assay and titrated using chick embryos with tenfold serial dilutions. Viral titers were determined according to the Reed and Muench method and expressed as the 50% egg infectious dose (EID<sub>50</sub>)/100 µL.

### Genome sequencing and phylogenetic analysis

Total viral RNA was extracted using TRIzol Reagent and reverse transcribed using Moloney murine leukemia virus reverse transcriptase (M-MLVRT, TaKaRa Co., China). AEV-specific primer pairs (Table 1) were designed based on the sequence of the Calnek-1143 strain (AJ225173). Seven DNA fragments corresponding to the complete ORF of AEV isolates were amplified using a high-fidelity pfu DNA polymerase (Q5™ High-Fidelity DNA polymerase, NEB), and the amplicons were sequenced commercially. To obtain the 5'- and 3'-termini of the AEV isolate genome, the 5'- and 3'-RACE (rapid amplification of cDNA ends) procedure was applied according to the manufacturer's instructions. Briefly, three specific primers were designed for the amplification of the 5' and 3' termini of the AEV isolate (Table 1). For amplification of the 5' terminus, cDNA was synthesized using the specific primer GSP1, purified, and subjected to PCR with the primer pairs Oligo(dt) and GSP2. For amplification of the 3' terminus, cDNA was synthesized using the primer Oligo(dt), purified, and subjected to PCR with the primer pairs Oligo(dt) and GSP3. The amplicons were sequenced commercially. Sequence assembly was carried out using the SeqMan program included with the DNASTAR software package (Madison, WI).

Phylogenetic analysis was performed based on the ORF nucleotide sequence of the GDt29 and GD-S-29 isolates from this study as well as other strains with nucleotide sequences available in the GenBank database (shown in

**Table 1** Primers used for genome amplification in this study

Primer	Sequence (5'-3')	Position	Size (bp)
AE1F	TTCGACCCAACCCATACT	37-54	1002
AE1R	TGGCACCTTCATCCTTACA	1021-1039	
AE2F	AAGGTTTGGCTTGAT	862-877	1599
AE2R	GGCACCTACCGATGTT	2446-2461	
AE3F	AAGGAACCACAGGGAC	2018-2033	1478
AE3R	CACCAAATCAAGAAAAT	3479-3496	
AE4F	CACATCGCTCACCAAA	3217-3232	858
AE4R	TCCATCCCAGAAGTCC	4060-4075	
AE5F	TGAGTTGCGTGCTGTT	3844-3859	1595
AE5R	CATTGTGCTCGTGCT	5424-5439	
AE6F	GAGGTTACCCCTTGTT	5161-176	1100
AE6R	GGCTCCATTATTCTATC	6245-6261	
AE7F	GTCCCACTAAGTTGTT	5752-5768	1280
AE7R	TTGGAATGTAGAGCC	7015-7032	
GSP1	CTCTGGTCTACCGTTGTCAG	616-635	
GSP2	GGTGTCTGTGCCGTGTG GGTA	570-589	
GSP3	CGATCAGGGCCAGCATAAG	6792-6812	

**Table 2** Previous AEV isolates used in this study

Strain	GenBank no.	Country	Year
Calnek-1143	AJ225173	UK	1960
L <sub>2</sub> Z	AY275539	China	2004
Van Reokel	AY517471	China	2004
204C	KF979338	Hong Kong	2013
Pf-CHK1	KT880668	Hungary	2015

Table 2). A phylogenetic tree based on the ORF sequences was constructed by the neighbor-joining (NJ) method using MEGA 5.0 software. The robustness of the phylogenetic analysis was determined by bootstrap analysis with 1000 replications.

### Pathogenicity analysis of the GDt29 isolate

To assess the pathogenicity of the GDt29 strain in chicken embryos, a total of fifteen 6-day-old SPF chick embryos were randomly divided into three groups (five embryos per group). The embryos in groups I and II were inoculated with the AEV strains GDt29 and Van Reokel, respectively, via the yolk sac route and the embryos in group III were inoculated with PBS as a mock infection control. Clinical signs of disease were monitored daily using the candling method. All of the embryos were sacrificed to observe their development at 10 dpi.

To assess the pathogenicity of the GDt29 strain in SPF chicks, a total of fifteen 1-day-old SPF chicks were randomly

divided into three groups (five chicks per group). The SPF chicks in group I were inoculated with GDt29 via the intracerebral inoculation route, and birds in group II were inoculated with Van Reokel via the intracerebral inoculation route as the positive infection control. The SPF chicks in group III were inoculated with PBS as a mock infection control. Clinical signs of disease and mortality were monitored daily. All of the chicks were humanely euthanized for tissue collection at 14 days postinfection (dpi). Pathological tissues, including the cerebrum, cerebellum, spinal cord and pancreas, were fixed by immersion in 10% neutral-buffered formalin, routinely processed, embedded in paraffin, sectioned (4 µm thick), and stained with hematoxylin and eosin (H&E) according to standard protocols. Pathological changes were examined by light microscopy.

### Safety of the AEV attenuated vaccine

Vaccine preparation was performed with AEV GDt29 strain and stabilizing buffer in a ratio of 1:1. The stabilizing buffer was composed of 10% of skim milk, 5% sucrose, and 85% distilled water, and equal volumes of GDt29 ( $10^{5.5}/0.1$  mL) virus and stabilizing buffer were mixed uniformly and kept as the candidate vaccine. The viral titer in the attenuated AEV vaccine was determined by the Reed and Muench method and expressed as the 50% egg infectious dose ( $EID_{50}$ )/100 µL. Safety testing was conducted according to the guidelines of the CVP 2010 Edition Volume III [4, 16]. Briefly, ten 7-day-old SPF chicks were vaccinated with the attenuated AEV vaccine, and clinical signs of disease and mortality were observed daily. All of the chicks were humanely euthanized for tissue collection at 21 dpi.

### Antibody generation in SPF hens following vaccination

To evaluate the efficacy of the attenuated AEV vaccine, a total of forty 14-week-old SPF hens were randomly distributed into four groups (ten hens per group). Birds in group I were inoculated with GDt29 attenuated vaccine ( $10^3 EID_{50}$  per chick) via the oral route, birds in group II were inoculated with a freeze-dried live commercial vaccine (FATRO S.P.A Ozzano Emilia Bologna, Italy) as positive control according to the manufacturer's instructions, and birds in group III received an equal volume of PBS alone as a mock-vaccination control.

An additional group of five birds received GDt29 attenuated vaccine ( $10^3 EID_{50}$  inoculation per chick), while another five birds were inoculated with PBS. The birds in each of the four groups were maintained in isolators with negative pressure. At 1, 2, 3, 4, 5, 6, 7 and 8 weeks postinfection, blood samples were collected from the wing vein and processed to collect sera for detection of antibodies. AEV-specific

antibodies were detected using an Avian Encephalomyelitis Virus Antibody Test Kit (IDEXX).

## Challenge studies

To assess the neutralizing activity of the maternal antibodies in the progeny of the GDt29-vaccinated hens, a neutralization assay was performed as described previously [17, 18]. Briefly, all of the SPF hens in groups I, II and III were artificially inseminated and fertilized eggs were then collected from the GDt29-vaccinated hens, commercial vaccine-vaccinated hens, and mock-vaccinated hens. A total of sixty 6-day-old chicken embryos were divided into four groups (15 embryos per group, with five eggs from the GDt29-vaccinated hens, five eggs from the commercial vaccine-vaccinated hens, and five eggs from the mock-vaccinated hens). The embryos in three groups were inoculated with Van Reokel via the yolk sac route with a dose of 10 LD<sub>50</sub>, 1 LD<sub>50</sub> and 0.1 LD<sub>50</sub>, respectively, while the embryos in the remaining group were inoculated with PBS as a negative control. All of the embryos in these four groups were hatched at 37 °C and a relative humidity of 55% until the birds hatched. Clinical signs of disease and morbidity were monitored using the candling method, and the hatching rate was determined. Blood samples were collected from the 1-day-old progeny from the wing vein and processed to produce serum samples for detection of antibodies using an Avian Encephalomyelitis Virus Antibody Test Kit (IDEXX). To confirm the challenge infection, a specific primer pair (5'-ACTGGCAAGCTTGTGTGCTATG-3', 5'-CTCCACTCAGGCACACTGGAA-3') was designed based on the genome sequence of the Van Reokel strain (*vp3/vp1* gene) for RT-PCR. The challenge virus was re-detected in the unhatched embryos or 1-day-old progeny, using an RT-PCR assay and sequencing assay.

## Statistical analysis

Statistical analysis was performed with GraphPad Prism (version 5.0), and data were expressed as the mean and standard deviation. The statistical significance of the data was determined using one-factor analysis of variance (ANOVA) to evaluate differences between the experimental groups. Differences between groups were considered significant at  $p < 0.05$ .

## Results

### Virus isolation

In 2015, an epidemiological survey was performed to evaluate the AEV epidemic overall. A total of 667 clinical

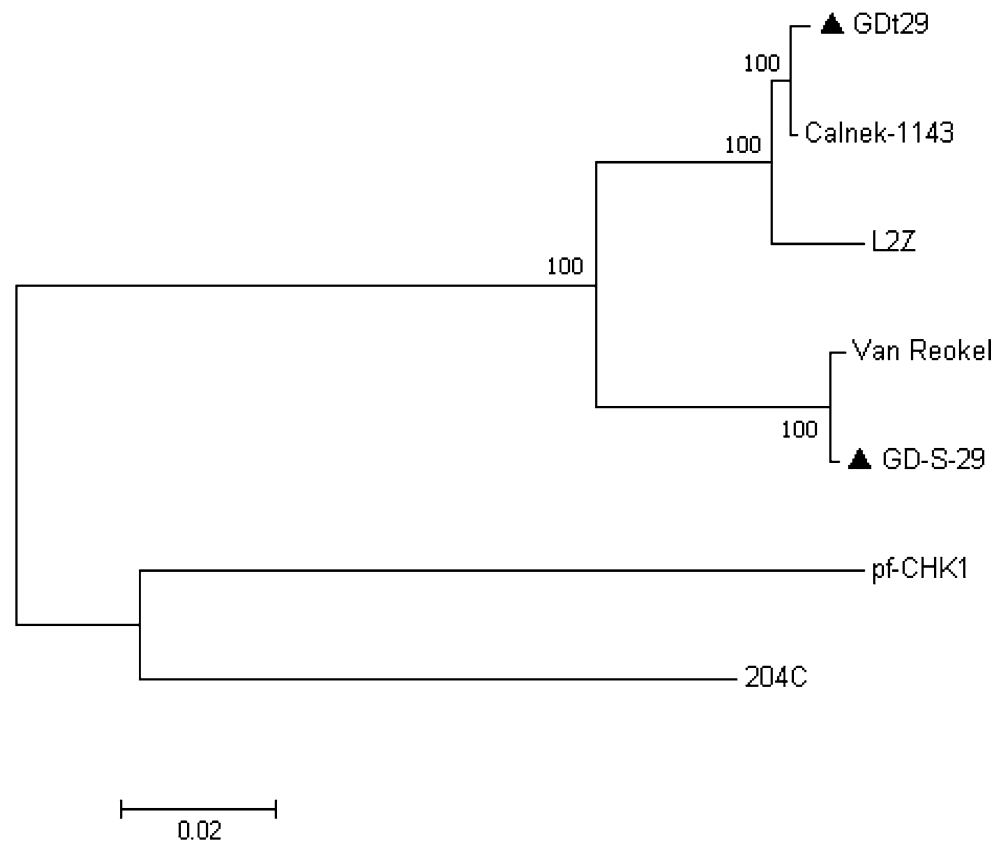
samples were collected from commercial chicken farms and tested using an RT-PCR assay. Of these, seven were positive for AEV. Virus isolation and purification were performed using SPF chick embryos. One AEV strain, designated as GDt29, was isolated from the pancreas and intestine tissue of birds that were collected from a chicken farm in Hunan province, and the other AEV strain, designated as GD-S-29, was isolated from the brain tissue of diseased birds that were collected from a chicken farm in Jiangxi province. This indicated that AEV is present in the southern Chinese poultry industry.

### Genome sequence and phylogenetic analysis of the GDt29 isolate

The complete genomes of GDt29 and GD-S-29 were sequenced commercially. The genomic sequences of GDt29 and GD-S-29 have been deposited in the GenBank database under the accession numbers MF620096 and MF179107, respectively. The complete genome of GDt29 is 7032 nt long, and the lengths of the 5'-untranslated region (UTR) and the 3'-UTR are 494 nt and 136 nt, respectively. The complete genome of GD-S-29 is 7031 nt in length, and the lengths of the 5'-UTR and 3'-UTR are 493 nt and 136 nt, respectively. Both viral genomes contain a large open reading frame (ORF), which is 6402 nt long and encodes 2134 aa, with no insertions or deletions in the coding regions.

A multiple sequence alignment based on the complete genome sequences of GDt29, GD-S-29 and other strains with sequences available in the GenBank database was made using the Clustal X program. The alignment showed that the genome sequence of the GDt29 isolate is the same length as those of the Calnek-1143 and L<sub>2</sub>Z strains but 1 nt longer than those of the pf-CHK1 and Van Reokel strains, and 77 nt longer than that of the 204C strain. The genome sequence of the GD-S-29 isolate is the same length as those of the pf-CHK1 and Van Reokel strains. Most of the differences in the lengths of the genome sequences occurred in the 5'-UTR and 3'-UTR. Compared to the sequence of Calnek-1143, 71 nucleotide differences were found in the genome of GDt29, and 335 in GD-S-29, causing 21 and 30 amino acid changes, respectively. Compare to the sequence of Van Reokel strain, 397 nucleotide differences were found in the genome of GDt29, and 24 in GD-S-29, causing 86 and eight amino acid changes, respectively. Among the nucleotide differences, 11 and 5 were unique to the genomes of GDt29 and GD-S-29, respectively. Most of these mutations were located in genes for the structural proteins (VP1, VP2 and VP3), which are prone to variation. Phylogenetic analysis showed that the GDt29 isolate is closely related to the Calnek-1143 and L<sub>2</sub>Z strains, while GD-S-29 is closely related to the Van Reokel strain Fig. 1. The detection of AEV isolates (GDt29 and GD-S-29) suggests that AEV is prevalent in China.

**Fig. 1** Phylogenetic analysis of the GDt29 isolate based on the nucleotide sequence of the complete ORF. The phylogenetic tree was constructed using MEGA 5.0 software. Bootstrap values obtained from 1,000 replicates are shown at the major nodes. The isolates identified in this study are indicated by solid triangles



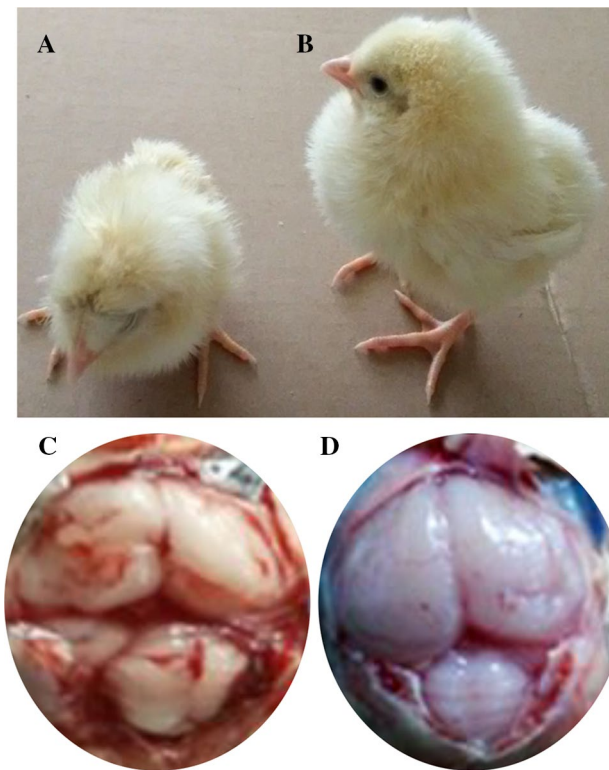
### Pathogenicity of the GDt29 strain in SPF eggs and chicks

Because GDt29 was isolated from birds that showed no obvious symptoms, whereas GD-S-29 was isolated from diseased birds, we speculated that GDt29 might possess lower virulence than GD-S-29. To test this hypothesis, we inoculated 1-day-old SPF chicks with GDt29 or GD-S-29 via the intracerebral inoculation route. Interestingly, GD-S-29-infected-chicks exhibited typical symptoms, including head droop, lassitude, ataxia and paralysis (Fig. 2A), while no GDt29-infected chicks exhibited any typical clinical symptoms of this disease (Fig. 2B), although the virus was still detected in the cerebrum. Anatomic analysis showed that hemorrhage and shrinkage had occurred in the brains of the GD-S-29-infected chickens (Fig. 2C), while brain development was normal in GDt29-infected chickens (Fig. 2D). These data suggest that GDt29 possesses lower pathogenicity than GD-S-29. Therefore, we selected the GDt29 strain for further investigation.

To assess the pathogenicity of the GDt29 strain in chicken embryos, 6-day-old fertilized SPF eggs were inoculated via the yolk sac route with the GDt29 strain, the Van Reekel strain as a positive control, or PBS as a negative control, and clinical signs of disease were monitored by candling and necropsy. We found that the original isolate GDt29 did not

cause any lesions in embryos, while the Van Reekel strain caused an obvious development disorder (Fig. 3). Next, GDt29 was serially passaged up to 15 times by inoculating 6-day-old fertilized SPF eggs by the yolk sac route to evaluate the replication characteristics of this isolate. The EID<sub>50</sub> of the original GDt29 isolate and the viruses obtained after the 5th passage, the 10th passage, and the 15th passage was 10<sup>5.5</sup>/0.1 mL, 10<sup>5.5</sup>/0.1 mL, 10<sup>5.55</sup>/0.1 mL, and 10<sup>5.5</sup>/0.1 mL, respectively.

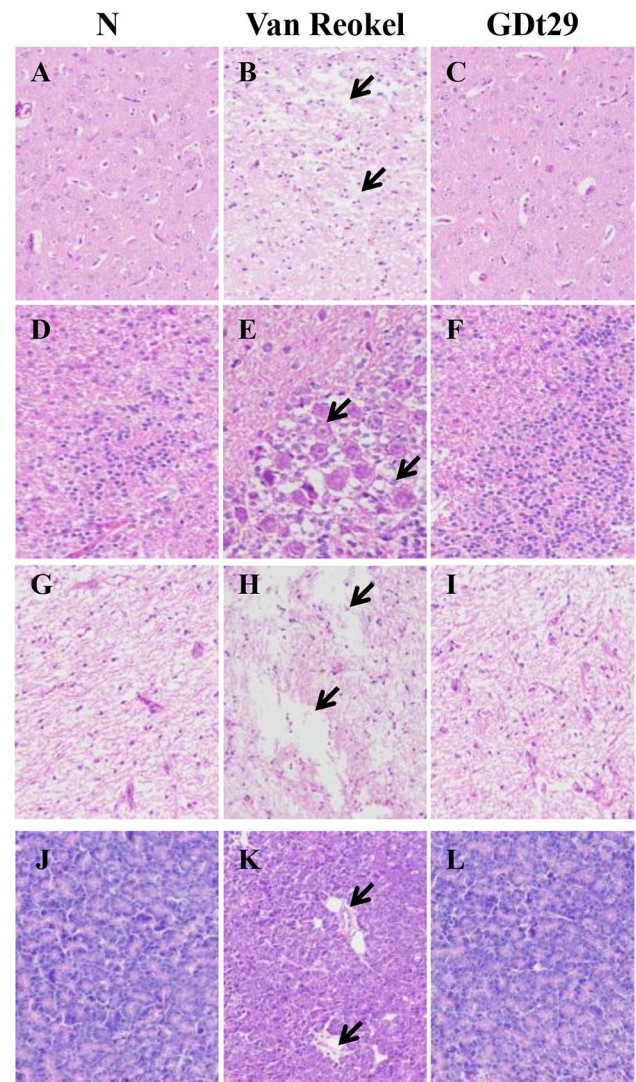
To evaluate whether the GDt29 strain could cause lesions in SPF chicks, we inoculated 1-day-old SPF chicks with the GDt29 strain or the Van Reekel strain as a control via the intracerebral inoculation route. Histopathologic analysis showed that the GDt29-infected chicks did not exhibit any lesions, while the Van Reekel-infected chicks had typical lesions in the cerebrum, cerebellum, spinal cord and pancreas. In the Van Reekel-infected chicks, lymphocytic perivascular infiltration and central chromatolysis was observed in large neurons of the cerebrum (Fig. 4A-C), necrosis of Purkinje cells occurred in the cerebellum, gliosis was observed in the molecular layer and central cerebellum (Fig. 4D-F), a loss of neurons was seen in the spinal cord (Fig. 4G-I), and there was focal necrosis and inflammatory cell infiltration in the pancreas (Fig. 4J-L). These data suggest that the GDt29 strain has low virulence and could be considered a candidate AEV vaccine strain.



**Fig. 2** Pathogenicity comparison of GDt29 and GD-S-29. One-day-old SPF chicks were inoculated with GDt29 or GD-S-29 via the intracerebral route. Clinical symptoms were observed, and anatomic analysis was performed. **A.** Clinical symptoms of a GD-S-29-infected chick based on appearance. **B.** Clinical symptoms of a GDt29-infected chick based on appearance. **C.** Brain development of a GD-S-29-infected chick. **D.** Brain development of a GDt29-infected chick



**Fig. 3** Pathogenicity of the GDt29 isolate in chicken embryos. Six-day-old SPF chicken embryos were inoculated with PBS (left), Strain Van Reokel (middle), or GDt29 (right). Embryo development was observed at 10 dpi



**Fig. 4** Histological changes in SPF chicks infected with the GDt29 strain. One-day-old SPF chicks were inoculated with PBS (left), strain Van Reokel (middle), or GDt29 (right). Pathological tissues were fixed by immersion in 10% neutral-buffered formalin, routinely processed, embedded in paraffin, sectioned (4  $\mu$ m thick), and stained with hematoxylin and eosin. Histological changes were examined by light microscopy. **(A-C)** Lymphocytic perivascular infiltration and central chromatolysis in large neurons of the cerebrum. **(D-F)** Necrosis of Purkinje cells in the cerebellum and gliosis in the molecular layer and central cerebellum. **(G-I)** Loss of the neurons reduced and focal lesions in the spinal cord. **(J-L)** Focal necrosis and inflammatory cell infiltration in the pancreas

### Safety of the attenuated AEV vaccine

The virus in the candidate vaccine was titrated according to the Reed and Muench method, and the  $EID_{50}$  of GDt29 was found to be  $10^{5.0}/0.1$  mL. To assess its safety, ten 7-day-old SPF chicks were inoculated with this vaccine, and after inoculation, all of the birds appeared to be normal, with no typical clinical symptoms, and no deaths

occurring, indicating that the GDt29 attenuated vaccine is safe.

### Humoral response induced by the GDt29 attenuated vaccine in SPF hens

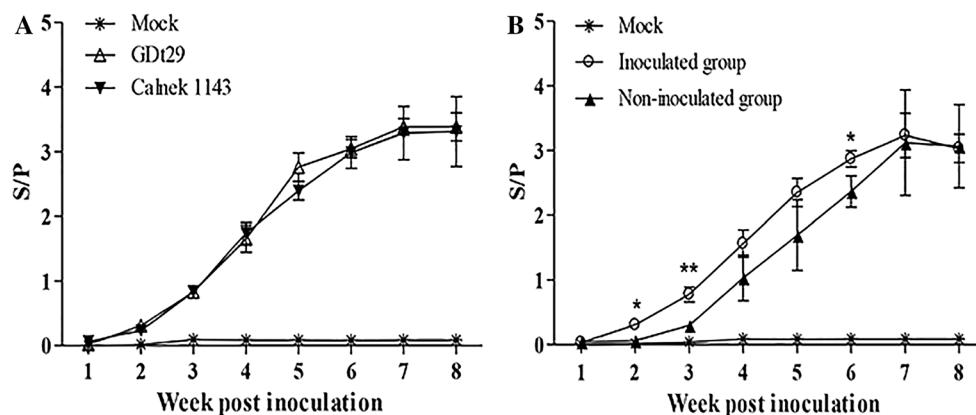
To assess the generation of antibodies after vaccination with the GDt29 attenuated vaccine, a total of thirty 14-week-old SPF hens (10 hens per group) were inoculated with GDt29 attenuated vaccine, commercial vaccine, or PBS (mock-vaccinated group). Similar to the mock-vaccinated group, no birds in the GDt29-immunized group or the commercial-vaccine-immunized group displayed apparent signs of disease. Specific antibody titers against AEV were determined at 1, 2, 3, 4, 5, 6, 7 and 8 weeks postinfection. As shown in Fig. 5A, no specific antibody against AEV was detected in the mock-vaccinated birds, while all the birds in the GDt29-immunized group and in the commercial-vaccine-immunized group showed an increase in the antibody titers at 2 weeks postinfection and maintained a high level of antibody 6 weeks after infection ( $S/P > 3.0$ ). The difference in antibody titers elicited by the GDt29 attenuated vaccine and the commercial vaccine was not statistically significant ( $p > 0.05$ ), indicating that the GDt29 attenuated vaccine and the commercial vaccine have similar antibody-inducing ability.

GDt29-vaccinated and contact-exposed hens were bred together in an isolator and tested for specific antibodies against AEV. As expected, the GDt29-vaccinated and contact-exposed hens both showed an increase in antibody titers after 4 weeks and developed high titers against AEV ( $S/P > 2.8$ ) at 7 weeks post-inoculation. The antibody titers in the GDt29-vaccinated hens was significantly higher than that in the contact-exposed hens at the early stage ( $P < 0.05$ ), but there was no significant difference in antibody titers after 7

weeks. The contact-exposed birds developed antibodies later than that of the GDt29-vaccinated birds, suggesting horizontal transmission of the GDt29 attenuated vaccine (Fig. 5B). Neither the GDt29-vaccinated nor contact-exposed birds developed any clinical signs of disease. These data suggest that the GDt29 attenuated vaccine confers strong humoral immunity in hens against AEV.

### Protection against challenge with AEV

To assess the protective efficacy of the GDt29 attenuated vaccine against challenge with AEV, the neutralizing activity of maternal antibodies was measured. Embryos in eggs laid by GDt29-vaccinated hens, commercial vaccine-vaccinated hens, and mock-vaccinated hens were inoculated with the heterologous strain Van Reokel and hatched under the proper conditions. The hatching rate of the eggs from the GDt29-vaccinated hens and the commercial-vaccine-vaccinated hens was 100% at an inoculation dose of 0.1 LD<sub>50</sub> or 1 LD<sub>50</sub>. Furthermore, all of the eggs from the GDt29-vaccinated hens hatched successfully at an inoculation dose of 10 LD<sub>50</sub>, and all of the chicks were normal in appearance. However, one egg from the commercial-vaccine-vaccinated hens failed to hatch, and one chick showed clinical symptoms of AEV at an inoculation dose of 10 LD<sub>50</sub>. To measure shedding of the challenge virus, we employed the RT-PCR assay and virus isolation. Consistent with the high hatching rate and lack of clinical symptoms, no challenge virus was detected in the progeny of any of the GDt29-vaccinated hens, regardless of the inoculation dose, or of the commercial-vaccine-vaccinated hens at an inoculation dose of 0.1 LD<sub>50</sub> or 1 LD<sub>50</sub>. However, challenge virus was detected in one unhatched egg and one chick from commercial-vaccine-vaccinated

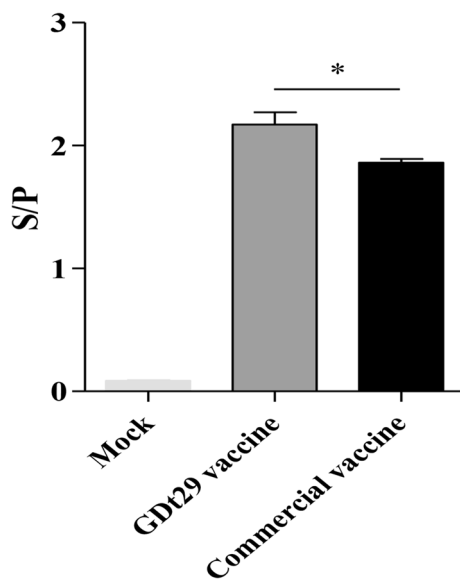


**Fig. 5** Humoral response to the GDt29 strain. SPF hens were randomly distributed into three groups and inoculated with commercial vaccine, GDt29 attenuated vaccine, or PBS. Serum samples were collected from the wing vein at 1, 2, 3, 4, 5, 6, 7 and 8 weeks post-

inoculation. Antibodies against AEV were detected using an Avian Encephalomyelitis Virus Antibody Test Kit (IDEXX). The results of all experiments are reported as the mean  $\pm$  S.D. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$

**Table 3** Protection efficiency of the GDt29 attenuated vaccine

Group	Dose	Number of eggs	Number hatching	Morbidity	Shedding of virus	Protection efficiency (%)
Commercial vaccine	10 LD <sub>50</sub>	5	4	2/5	2/5	60
	1 LD <sub>50</sub>	5	5	0/5	0/5	100
	0.1 LD <sub>50</sub>	5	5	0/5	0/5	100
GDt29	10 LD <sub>50</sub>	5	5	0/5	0/5	100
	1 LD <sub>50</sub>	5	5	0/5	0/5	100
	0.1 LD <sub>50</sub>	5	5	0/5	0/5	100
PBS	10 LD <sub>50</sub>	5	0	5/5	5/5	0
	1 LD <sub>50</sub>	5	1	5/5	5/5	0
	0.1 LD <sub>50</sub>	5	3	5/5	5/5	0



**Fig. 6** Maternal antibodies against AEV in progeny. All SPF hens in the GDt29-vaccinated group, commercial-vaccine-vaccinated group, and mock-vaccinated group were artificially inseminated, and 15 fertilized eggs (5 per group) were hatched at 37 °C at a relative humidity of 55%. Blood samples were collected from the wing vein of the 1-day-old progeny and processed to obtain sera for detection of antibodies using an Avian Encephalomyelitis Virus Antibody Test Kit (IDEXX). The results of all experiments are reported as the mean  $\pm$  S.D. \*,  $P < 0.05$

hens at an inoculation dose of 10 LD<sub>50</sub> (Table 3). These data suggest that the GDt29 attenuated vaccine possesses higher protective efficacy against AEV than the commercial vaccine.

Fifteen fertilized eggs from the remaining group hatched successfully after 21 days, and blood samples were collected from the wing vein and processed to obtain sera for antibody detection. As shown in Fig. 6, the progeny of GDt29-vaccinated hens had higher maternal antibody titers ( $S/P > 2.0$ ) than the chicks of

commercial-vaccine-vaccinated hens ( $S/P > 1.8$ ), while maternal antibody was undetectable in the offspring of mock-vaccinated hens.

## Discussion

AEV can be transmitted through the faecal-oral route and transmitted to embryos [9, 11, 13]. If vaccination is not used on poultry farms, this disease can cause huge economic losses. Vaccination of flocks is still an effective way to control and prevent this disease, and due to these effective immunization measures, widespread outbreaks of AEV have not occurred in recent years. As a consequence, AEV receives little attention in the poultry industry. In the present study, we performed an epidemiological survey of breeding chicken farms in southern China. Among the 667 clinical samples tested, seven were positive for AEV, and two AEV strains were isolated and designated as GDt29 and GD-S-29, respectively. This is consistent with previous reports [7, 12, 19], that AEV continues to be present in the poultry industry.

Like those of other picornaviruses, the genomes of GDt29 and GD-S-29 are composed of a large ORF, a 5'-UTR and a 3'-UTR. Both ORFs are 6402 nt long and encode a polyprotein of 2134 aa, with no insertions or deletions in the coding regions. The AEV VP1 protein is the major host-protective immunogen and is therefore important for diagnostics and vaccine development, and it has been observed that the *vp* genes mutate easily under immune selective pressure [2]. Consistent with these reports, the mutations observed in the present study mainly occurred in the *vp1* gene of the 11 mutations in the genome of GDt29, five occurred in the *vp1* gene, one occurred in the *vp2* gene and two occurred in the *vp3* gene. Of the five mutations in the genome of GD-S-29, two occurred in the *vp2* gene and two occurred in the *vp3* gene. More effort will be required to determine the effect of these mutations on the replication and immunogenicity of AEV.



Challenge experiments are essential for enhancing our understanding of AEV isolates. Therefore, we attempted to assess the pathogenicity of GDt29 and GD-S-29 using SPF chickens. Surprisingly, the original isolate GD-S-29 appeared to cause head droop, lassitude, ataxia and paralysis and induced hemorrhage and shrinkage in the brain. In contrast, no obvious typical clinical symptoms and no pathological lesions were observed in GDt29-infected chickens, indicating that GDt29 has low virulence in SPF chickens. Furthermore, the original isolate GDt29 did not cause any lesions in embryos. An ideal vaccine candidate strain should possess low virulence. These observations clearly show that the GDt29 strain has low virulence and could be considered a candidate vaccine strain against AEV.

It has been reported that AEV is constantly present in the poultry industry, and the most effective way to prevent AEV infection is vaccination of flocks [7, 10, 19, 20]. AEV vaccination is usually performed by administering a live, embryo-propagated virus by the natural route of infection in drinking water or by wing-web inoculation with an AEV strain of low pathogenicity. For a live candidate vaccine, safety testing is necessary. Therefore, we employed 1-day-old SPF chicks, 7-day-old SPF chicks and 14-week-old SPF hens to determine the safety of the candidate vaccine. Surprisingly, we found that none of the chickens inoculated with the GDt29 attenuated vaccine displayed any typical clinical signs of AE disease, suggesting that the GDt29 attenuated vaccine is safe for chickens.

It has been reported that some live attenuated viruses are more efficient than inactivated vaccines at stimulating the immune response in a durable manner, and live attenuated vaccines are less expensive than inactivated virus to produce because there is no requirement for an inactivation step and for an additional adjuvant to boost the immune response [15]. In addition to these advantages, the GDt29 attenuated vaccine can be given orally, reducing stress to the chickens. In the present study, 14-week-old hens were selected for vaccination because AEV infection of birds of this age does not cause any clinical signs but induces long-lasting immunity. As expected, GDt29 inoculation led to high levels of AEV-specific antibodies in the hens, suggesting that the required protective immune response has been achieved. Moreover, AEV-specific antibodies in the hens were detected steadily during laying, indicating long-term protection of the hens by the GDt29 vaccine.

It has been reported that AEV can be transmitted through the faecal-oral route [3]. We therefore kept mock-vaccinated hens with GDt29-vaccinated birds in an isolator with negative pressure in the present study. As expected, the contact-exposed chicks developed high antibody titers against AE virus after 3 weeks, and GDt29 virus was detected in these contact-exposed chicks after 10 days, indicating efficient horizontal transmission of GDt29. These data clearly show that

horizontal transmission of GDt29 confers immunity against AEV in chicken flocks.

Vertical transmission is an important route for AEV infection [11]. Vaccination prevents progeny from getting infected, and that prevents vertical transmission [2]. Because the GDt29 attenuated vaccine should also possess this characteristic, we attempted to measure the neutralizing activity of maternal antibodies in the present study. Interestingly, all the eggs from the GDt29-vaccinated hens and the commercial-vaccine-vaccinated hens were hatched successfully at an inoculation dose of 0.1 LD<sub>50</sub> or 1 LD<sub>50</sub>, indicating a high neutralizing activity in these eggs. However, the maternal antibodies in commercial-vaccine-vaccinated hens' eggs did not give adequate protection (only 60%) against AEV with an inoculation dose of 10 LD<sub>50</sub>, but the maternal antibodies in the GDt29-vaccinated hens' eggs were sufficient to allow all of the eggs to hatch successfully. Virus shedding was consistent with the hatching rate and clinical symptoms. AEV was detected in only two samples from commercial-vaccine-vaccinated hens at an inoculation dose of 10 LD<sub>50</sub>. Maternal antibody detection of the progeny showed that the progeny of GDt29-vaccinated hens had higher maternal antibody titers than those of the controls. These data indicate that the GDt29 attenuated vaccine confers immunity against AEV and provides stronger protection to the progeny than the commercial vaccine. The administration of GDt29 attenuated vaccine to breeding hens before eggs are laid can play an important role in preventing AEV infection.

The results of this study suggest that the GDt29 attenuated vaccine is safe and stimulates an immune response, exhibiting a fine balance between attenuation and immunogenicity. The GDt29 vaccine should be considered as a candidate vaccine to prevent AEV infection.

**Funding** This study was supported by National Key R&D Program of China (2017YFD0502001) and by Guangdong Province Science and Technology Plan Project (2015A020209137, 2016A050502042, 2016A020210125).

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no competing interests.

**Ethical approval** All animal experiments were approved by the Committee of the Ethics on Animal Care and Experiments at South China Agricultural University (approval ID: 201004152). All study procedures and animal care activities were conducted in accordance with the national and institutional guidelines for the care and use of laboratory animals.

## References

1. Toplu N, Alcigir G (2004) Avian encephalomyelitis in naturally infected pigeons in Turkey. *Avian Pathol* 33:381–386

2. Welchman DB, Cox WJ, Gough RE, Wood AM, Smyth VJ, Todd D, Spackman D (2009) Avian encephalomyelitis virus in reared pheasants: a case study. *Avian Pathol* 38:251–256
3. Berger RG (1982) An in vitro assay for quantifying the virus of avian encephalomyelitis. *Avian Dis* 26:534–541
4. Hauck R, Senties-Cue CG, Wang Y, Kern C, Shivaprasad HL, Zhou H, Gallardo RA (2017) Evolution of avian encephalomyelitis virus during embryo-adaptation. *Vet Microbiol* 204:1–7
5. Itakura C, Goto M (1975) Avian encephalomyelitis in embryos and abnormal chicks on the day of hatching—neurohistopathological observations. *Nihon Juigaku Zasshi* 37:21–28
6. Meroz M, Elkin N, Hadash D, Abrams M (1990) Egg drop associated with avian encephalomyelitis virus. *Vet Rec* 127:532
7. Liu Q, Yang Z, Hao H, Cheng S, Fan W, Du E, Xiao S, Wang X, Zhang S (2014) Development of a SYBR Green real-time RT-PCR assay for the detection of avian encephalomyelitis virus. *J Virol Methods* 206:46–50
8. Marvil P, Knowles NJ, Mockett AP, Britton P, Brown TD, Cavanagh D (1999) Avian encephalomyelitis virus is a picornavirus and is most closely related to hepatitis A virus. *J Gen Virol* 80(Pt 3):653–662
9. Calnek BW (1998) Control of avian encephalomyelitis: a historical account. *Avian Dis* 42:632–647
10. Yu XH, Zhao J, Qin XH, Zhang GZ (2015) Serological evidence of avian encephalomyelitis virus infection associated with vertical transmission in chicks. *Biologicals* 43:512–514
11. Miyamae T (1980) Horizontal transmission of a pancreas-passaged avian encephalomyelitis virus in chicks. *Am J Vet Res* 41:584–585
12. Senties-Cue CG, Gallardo RA, Reimers N, Bickford AA, Charlton BR, Shivaprasad HL (2016) Avian encephalomyelitis in layer pullets associated with vaccination. *Avian Dis* 60:511–515
13. Smyth JA, McNeilly F, Reilly GA, McKillop ER, Cassidy JP (1994) Avian encephalomyelitis following oral vaccination. *Avian Pathol* 23:435–445
14. Westbury HA, Sinkovic B (1978) The pathogenesis of infectious avian encephalomyelitis. 4. The effect of maternal antibody on the development of the disease. *Aust Vet J* 54:81–85
15. Minor PD (2015) Live attenuated vaccines: Historical successes and current challenges. *Virology* 479–480:379–392
16. Feng K, Xue Y, Wang J, Chen W, Chen F, Bi Y, Xie Q (2015) Development and efficacy of a novel live-attenuated QX-like nephropathogenic infectious bronchitis virus vaccine in China. *Vaccine* 33(9):1113–1120
17. Calnek BW, Jehnich H (1959) Studies on avian encephalomyelitis. II. Immune responses to vaccination procedures [J]. *Avian Dis* 3:225–239
18. Calnek BW, Taylor PJ, Sevoian M (1961) Studies on avian encephalomyelitis. V. Development and application of an oral vaccine. *Avian Dis* 5:297–312
19. Wei L, Chee L, Wei T, Liu J (2008) The VP1 protein of avian encephalomyelitis virus is a major host-protective immunogen that serves as diagnostic potential [J]. *J Virol Methods* 149(1):56–62
20. Taunde P, Timbe P, Lucas AF, Tchamo C, Chilundo A, Dos Anjos F, Costa R, Bila CG (2017) Serological evidence of avian encephalomyelitis virus and *Pasteurella multocida* infections in free-range indigenous chickens in Southern Mozambique. *Trop Anim Health Prod* 49(5):1047–1050