ORIGINAL ARTICLE

Phylogenetic analysis of infectious bronchitis virus circulating in southern China in 2016–2017 and evaluation of an attenuated strain as a vaccine candidate

Libin Chen[1] · Bin Xiang[1] · Yanfen Hong[1] · Qian Li[1] · Haoyun Du[1] · Qiuyan Lin[1] · Ming Liao[1] · Tao Ren[1] · Chenggang Xu[1]

Abstract

Avian infectious bronchitis (IB) is a highly contagious viral respiratory disease, caused by infectious bronchitis virus (IBV), that poses an important economic threat to the poultry industry. In recent years, genotypes GI-7, GI-13, and GI-19 have been the most prevalent IBV strains in China. However, in this study, we found that most IBV strains from southern China in 2016–2017 belonged to genotype GVI-1. This genotype, for which there is no vaccine, has been reported sporadically in the region. The GDTS13 strain, which caused severe IB outbreaks on the farms where it was isolated, was evaluated as a candidate vaccine strain. GDTS13 was serially passaged in specific-pathogen-free embryonated chicken eggs for 100 generations to produce GDTS13-F100. Safety testing indicated that GDTS13-F100 had no pathogenic effect on chickens. Additionally, GDTS13-F100 showed an excellent protective effect against GDTS13, with no clinical signs or virus shedding observed in immunized chickens challenged with the parent strain. These findings indicate that GVI-1 has become the most prevalent IBV genotype in southern China and that GDTS13-F100 may serve as an attenuated vaccine to protect against infection with this genotype.

✉ Tao Ren
rentao6868@126.com

✉ Chenggang Xu
chgangxu@scau.edu.cn

1 National and Regional Joint Engineering Laboratory for Medicament of Zoonosis Prevention and Control, Key Laboratory of Zoonosis Prevention and Control of Guangdong Province, Key Laboratory of Animal Vaccine Development of Ministry of Agriculture, College of Veterinary Medicine, South China Agricultural University, 483 Wushan Road, Tianhe District, Guangzhou 510642, People's Republic of China

Introduction

Because of its high morbidity, widespread distribution, and large associated economic losses, avian infectious bronchitis (IB) is considered as one of the most important infectious diseases in the commercial chicken industry [1, 2]. It is a highly contagious viral respiratory disease caused by infectious bronchitis virus (IBV), which was first described in North Dakota, USA, in the 1930s [3]. In chickens, IBV damages not only the respiratory system but also the bursa of Fabricius, digestive tract tissues, and the urogenital system, resulting in poor egg quality and even death [1, 4].

IBV is a coronavirus with an approximately 27-kb positive-sense single-stranded RNA genome that encodes four structural proteins, including the spike glycoprotein (S) and the envelope (E), membrane (M), and nucleocapsid (N) proteins, as well as four non-structural accessory proteins, 3a, 3b, 5a, and 5b [5]. The RNA-dependent RNA polymerase of this virus has limited proofreading ability, and mutations and recombination events that occur during replication of the IBV genome lead to the abundant genetic diversity observed for this virus [6, 7]. Therefore, new genotypes and serotypes of IBV are continually emerging worldwide.

The S protein has approximately 1170 amino acids and is cleaved into two subunits of nearly equal mass, S1 and S2, after translation [8]. Unlike the conserved S2 subunit, the S1 subunit contains the virus-neutralizing epitope of IBV and is closely associated with the virulence and tissue tropism of the virus [9, 10]. Nucleotide sequences are most heterogeneous in the S1 gene, which is prominent in three hypervariable regions (HVRs) (amino acids 38–67, 91–141, and 274–387) [11, 12]. Therefore, to determine the genetic types of IBV, nucleotide sequences of complete or partial S1 genes are commonly analyzed. Among the numerous classifications based on S1 analysis, a new scheme has been proposed that established 35 lineages of IBV, including seven genotypes (GI–GVII), with many additional inter-lineage recombinants [13, 14].

Since it was first isolated and identified in 1982, circulation of IBV has been observed in China [15]. Although vaccines based on the 4/91 strain or Massachusetts (Mass) strains such as H120 and H52 have been used for many years, IB continues to be a serious threat to China's poultry industry [16, 17]. In recent years, genotypes GI-7, GI-13, and GI-19, also known as TW-type, 4/91-type, and QX/LX4-type viruses, respectively, have been the major IBV strains isolated in China [17–19]. However, multiple genotypes of IBV, including GVI-1, have been detected sporadically across the country [20]. Specifically, genotype GVI-1 has been isolated in South Korea and China since 2007 and is a respiratory pathogen according to available data [21, 22]. Most GVI-1 strains isolated in China are concentrated in the southern region [18, 21].

In this study, 16 IBV strains were isolated from tissue samples of chickens in Guangdong province of southern China in 2016–2017. Phylogenetic and genetic analysis showed that 10 of the isolates belong to genotype GVI-1, indicating that it has been the main genotype of IBV circulating in Guangdong in recent years. We chose one strain, named GDTS13, that caused severe IB outbreaks on farms where it was isolated, for evaluation as a candidate vaccine strain. The GDTS13 strain was attenuated by continuous passage for 100 generations in specific-pathogen-free (SPF) embryonated chicken eggs. Finally, we evaluated the safety of attenuated GVI-1 IBV GDTS13-F100 and the protective efficacy of this strain against challenge with various GVI-1 IBV strains. Most tests were performed in accordance with the requirements of the Veterinary Pharmacopoeia of the People's Republic of China (CVP) 2010 Edition and OIE standards (https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.03.02_AIB.pdf).

Materials and methods

Embryonated chicken eggs and chicks used in experiments

Embryonated SPF White Leghorn eggs and chicks were purchased from Guangdong Wens Dahuanong Biotechnology Co., Ltd. (Guangzhou, China). All animal experiments were conducted under the guidance of the South China Agricultural University Institutional Animal Care and Use Committee and Association for Assessment and the Accreditation of Laboratory Animal Care International-Accredited Facilities. The protocol was reviewed and approved by the Committee on the Ethics of Animal Experiments of South China Agricultural University (approval ID no. SCXK (Guangdong) 2018-0027).

Virus isolation and RNA extraction

Sixty-two tissue samples, including the kidneys, tracheas, and lungs, were collected from broiler chickens suspected of being infected with IBV on poultry farms in Guangdong province in 2016–2017. These chickens exhibited clinical signs, including lethargy, feather ruffling, neurologic symptoms, and sneezing. All of the sick chickens had been immunized against Mass strains such as H120 and H52. The samples were cut and ground, frozen and thawed three times, suspended in sterile phosphate-buffered saline (PBS) containing 200 U/mL penicillin and 200 μg/mL streptomycin, and then centrifuged at 10,000 rpm for 5 min at 4℃. The supernatants were collected and inoculated into 10-day-old SPF chicken embryonated eggs by the allantoic cavity route. Embryos that died within 24 h after inoculation were discarded. Infectious allantoic fluid was collected at 60 h post-inoculation and stored at –80℃ until further processing. RNA was extracted from the allantoic fluid using a RaPure Viral RNA/DNA Kit (Magen, Guangdong, China) according to the manufacturer's instructions. IBV infection was verified by reverse transcription polymerase chain reaction (RT-PCR) using a PrimeScript One Step RT-PCR Kit (Takara, Shiga, Japan) for the M gene. Passages GDTS13-F40, F60, F80, and F100 were obtained by continuous passage of GDTS13 through 10-day-old SPF chicken embryonated eggs for 40, 60, 80, and 100 passages, respectively. The median embryo infectious doses (EID_{50}) of the strains were calculated as described by Reed and Muench [23].

RT-PCR and sequencing

Pairs of primers targeting the S1 gene and 17 fragments of the complete IBV genome (Supplementary Table 1) were

designed to amplify, clone, and sequence the genomes of isolates using Oligo 7. The genomic sequence of the GDTS13 strain and sequences of the S1 gene of other isolates were verified by RT-PCR using a PrimeScript One Step RT-PCR Kit Ver. 2 (Takara). Target products obtained by RT-PCR were purified and recovered using a Gel Extraction Kit (Omega Bio-tek, Norcross, GA, USA). The RT-PCR products were ligated into the pMD19-T vector (Takara) and used to transform DH5α competent cells (Tiangen, Beijing, China). Plasmids positive for these products were sequenced by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China).

Genetic and phylogenetic analysis of the S1 gene

All IBV S1 gene nucleotide and protein sequences were aligned using EditSeq (DNASTAR, Madison, WI, USA) and compared with 156 reference IBV strains (~1620 bp) representing 35 well-established lineages and seven genotypes published in GenBank, including all 49 strains of the GVI-1 genotype (updated March 1, 2020), using the ClustalW multiple alignment algorithm in MegAlign (DNASTAR). S1 gene nucleotide sequences were phylogenetically analyzed using the maximum-likelihood method in MEGA version 10.0.2 (https://www.megasoftware.net) and the Tamura-Nei model with 1000 bootstrap replicates.

Virus cross-neutralization testing

Immune sera were prepared as described previously [24] against vaccine strains H120, 4/91, M41, Holte, and Conn46. The immune sera were serially diluted twofold with sterile PBS, and each dilution was mixed with an equal volume of a suspension containing 200 EID_{50} of each strain. The mixtures were incubated at 37℃ for 1 h. Ten-day-old SPF chicken embryonated eggs were inoculated with the mixture via the allantoic cavity route and incubated at 37℃ for 7 days. The serum neutralization titer was the highest dilution of serum that protected more than 50% of the chicken embryos dwarfing or death following vaccine challenge.

Evaluation of GDTS13 strain pathogenicity in SPF chickens

Allantoic fluid containing GDTS13 was tested for other pathogens including bacteria, mycoplasma, Newcastle disease virus, avian influenza virus, infectious laryngotracheitis virus, avian reovirus, infectious bursal disease virus, Marek's disease virus, avian reticuloendothelial hyperplasia virus, avian lymphoblastic leukemia virus, avian encephalomyelitis virus, chicken pox virus, and avian adenovirus. To evaluate the pathogenicity of the GDTS13 strain, 100 1-day-old SPF chickens were randomly divided into two groups (n = 50), with chicks in one group challenged with allantoic fluid containing the GDTS13 strain at a dose of 10^5 EID_{50}/200 µL per chick via the intraocular (100 µL) and intranasal (100 µL) routes and 50 chicks inoculated with 200 µL of sterile PBS via the same routes as controls. Ten chicks in each group were randomly selected and sacrificed on days 3, 5, 7, and 9 postinfection. Trachea, lung, kidney, and glandular stomach tissues were collected from the chickens and ground for virus isolation as described above. All chickens were maintained in isolators under negative pressure and observed daily for illness or death throughout the experimental period. Any chick showing clinical signs was considered diseased.

Safety testing of different generations of the GDTS13 strain

Fifty 1-day-old SPF chickens were randomly divided into five groups (n = 10), four of which (groups A, B, C, and D) were challenged with allantoic fluid containing the GDTS13 strain at a dose of 10^5 EID_{50}/200 µL per chick at various passages (GDTS13-F40, -F60, -F80, and -F100, respectively) via the intraocular and intranasal routes. Ten chicks were inoculated with 200 µL of sterile PBS via the same route as a control group (group E). All chicks were sacrificed at 15 days postinfection, and the trachea, lung, kidney, and glandular stomach tissues were collected and ground for virus isolation. All chicks were maintained in isolators under negative pressure and observed daily for illness or death throughout the experimental period. Any chick showing clinical signs was considered diseased.

Determination of the protective efficacy of GDTS13-F100 against challenge with genotype GVI-I field strains

Forty 1-day-old SPF chickens were randomly divided into four groups of 10 chickens each and housed in different isolators. Chicks in group A were inoculated with GDTS13-F100 at a dose of $10^{3.5}$ EID_{50}/100 µL per chick via the intraocular and intranasal routes. Chicks in group B were intraocularly and intranasally vaccinated with one dose of H120 (Boehringer Ingelheim, Ingelheim am Rhein, Germany) as recommended by the manufacturer. Chicks in groups C and D were inoculated with 100 µL of sterile PBS via the same routes. At 14 days post-immunization, chicks in groups A, B, and C were challenged with allantoic fluid containing the GDTS13 strain at a dose of 10^5 EID_{50}/200 µL per chick via the intraocular and intranasal routes, whereas chicks in group D were inoculated with 200 µL of sterile PBS via the same route to as controls. All chicks were observed daily for death and clinical signs attributable to IBV infection for 14 days. Any chick showing clinical signs was considered diseased. Oropharyngeal and cloacal swabs

were obtained at 3, 5, 7, and 9 days postinfection to detect IBV shedding from chickens. Swabs were placed in 1 mL of sterile PBS containing 200 U of penicillin and 200 μg of streptomycin per mL, and each fluid was inoculated into five SPF chicken embryonated eggs. The allantoic fluid was tested for IBV by RT-PCR as described above.

Results

Virus isolation

Sixty-two tissue samples were collected from chickens suspected of being infected with IBV on poultry farms in Guangdong province in 2016–2017. As shown in Fig. 1, 16 IBV strains were isolated from the tissue samples of chickens obtained in different cities. Huizhou showed the highest rate of isolation from tissue samples (9/20, 45%), followed by Yunfu (2/6, 33.3%) and Jiangmen (5/18, 27.8%).

IBV was not isolated from tissue samples from Qingyuan (0/6), Foshan (0/4), or Guangzhou (0/8). Supplementary Table S2 shows information on each isolated IBV strain.

Phylogenetic analysis and sequence comparison based on complete S1 genes of 16 IBV strains

As shown in Figure 2A, one of the IBV isolates (TJ402) clustered with the GI-13 lineage of IBV, and four isolates (TJ401, GDJM502, GDJM505, and GDSB1220) were classified as lineage GI-19, which includes the QX-type strain, the main epidemic strain circulating in China in recent years. Additionally, 10 of the IBV isolates belonged to lineage GVI-1, which comprises isolates collected in China and Korea after 2007. Combined with the data of this study, the majority of the GVI-1 strains published in GenBank were isolated in south China (34/59, 57.6%) (Fig 2B).
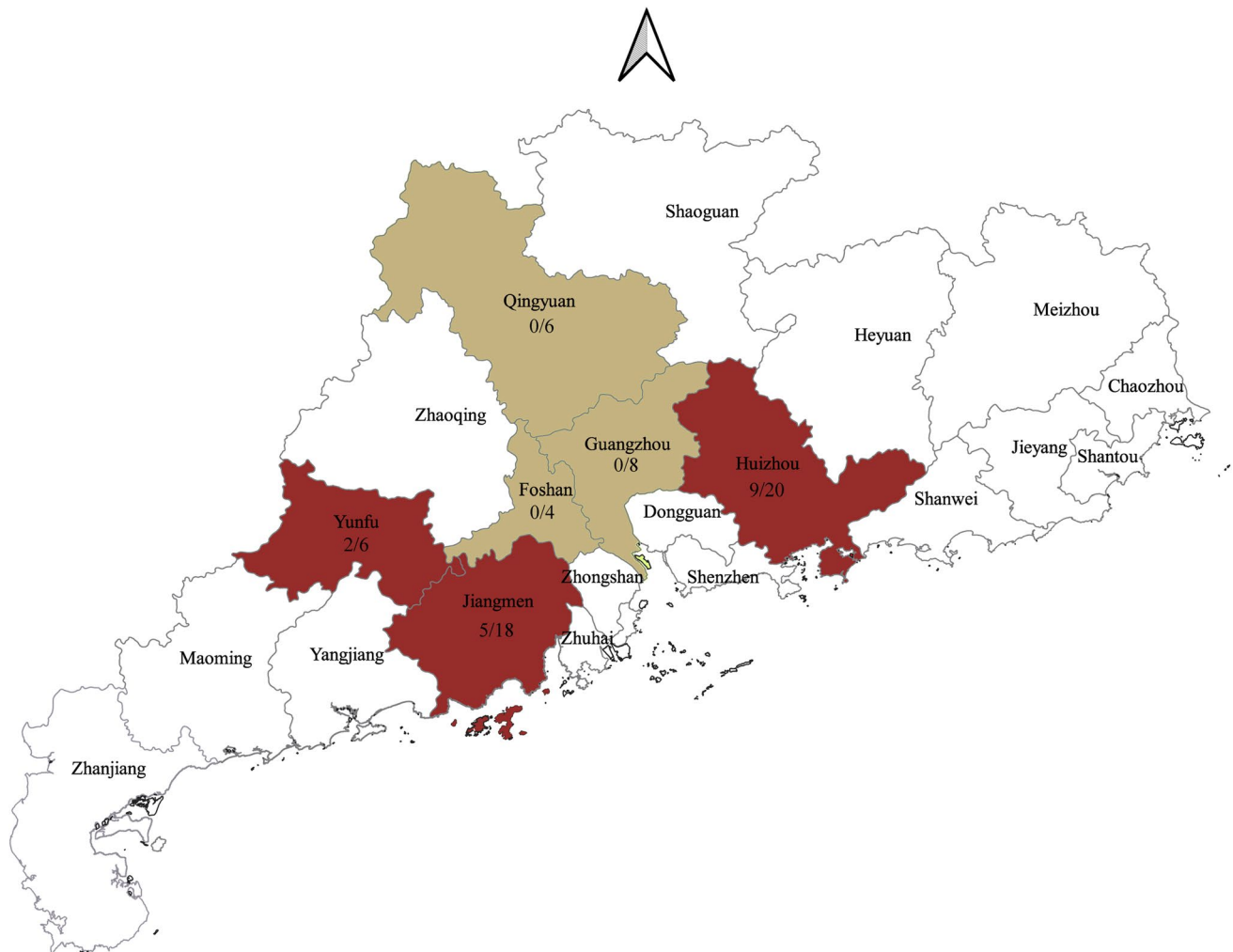

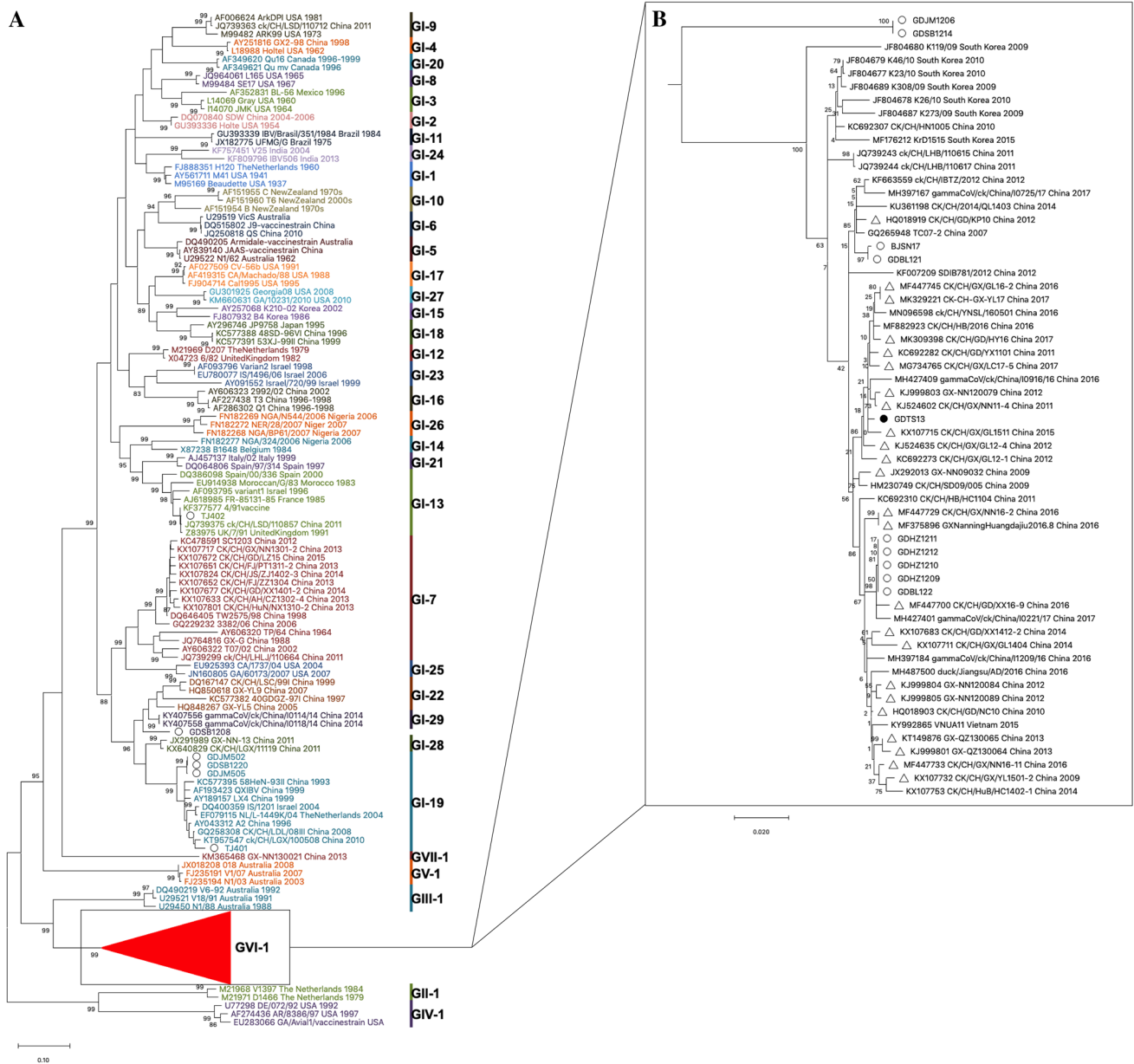**Fig. 1** Sources of IBV isolates from Guangdong province

Fig. 2 Phylogenetic relationships between 16 isolates and 156 reference strains based on complete S1 gene sequences. The tree was created by the maximum-likelihood method in MEGA version 10.0.2 using the Tamura-Nei model and 1000 bootstrap replicates. Twenty-four reference GVI-1 strains from southern China are indicated by triangles, whereas the 15 isolates from this study are indicated by open circles, and GDTS13 is indicated by a solid circle. (A) Strains of the same lineages are shown in the same color. (B) Ten isolates are shown with all GVI-1 strain sequences (49 isolates) published in GenBank (updated to March 1, 2020).

Analysis of S1 structural proteins

The GDTS13-F100 S1 gene was found to contain four mutations resulting in three amino acid changes (Table 1). The amino acid substitution R289K was located in HVR2 of the S1 protein. The nucleotide and amino acid sequences of GDTS13-F80 and GDTS13-F100 were both 100% identical, indicating that GDTS13 was genetically stable after serial passage in SPF chicken embryonated eggs.

Antigenic characterization of strain GDTS13

Strain GDTS13, one of the 10 isolates classified as belonging to lineage GVI-1, caused a severe IB outbreak on farms where it was isolated. After isolation and purification, no other pathogens were detected in the allantoic fluid containing strain GDTS13. To evaluate cross-neutralization reactivity between GDTS13 and vaccines that are widely used in China, five vaccine strains (H120, 4/91, Conn46, M41,

and Holte) and GDTS13 were used to prepare immune sera. Serum antibodies against strain GDTS13 and five vaccine strains showed a high titer, suggesting that these sera were appropriate for use in a neutralization test. As shown in Table 2, the neutralization titers of antibodies against the five vaccine strains were all less than 8 against strain GDTS13, indicating that serum antibodies produced in response to the vaccine strains did not effectively neutralize this strain.

Evaluation of the pathogenicity of strain GDTS13 in SPF chickens

All chickens in the GDTS13-infection groups exhibited previously described clinical manifestations of IBV [17, 25, 26], including lethargy, feather ruffling, neck shrinkage, head shaking, sneezing, and diarrhea. GDTS13 virus replicated

in the trachea, lung, kidney, and glandular stomach tissues from days 3 to 9 postinfection, whereas the virus was only detected in the liver tissue from days 3 to 5 postinfection. Replication of strain GDTS13 was most obvious in the trachea and lungs (Fig 3).

Safety testing of different passages of strain GDTS13

To evaluate whether the pathogenicity of GDTS13 was attenuated by serial passaging, SPF chickens were challenged with the GDTS13 strain from different passages. Fifty percent of SPF chickens inoculated with GDTS13-F40 exhibited clinical manifestations, including lethargy, feather ruffling, neck shrinkage, head shaking, and sneezing, whereas only 20% of SPF chicken inoculated with

Table 1 Amino acid changes observed in the S1 protein of different generations of IBV genotype GVI-1 strain GDTS13

	33							289							397						
GDTS13-F1	Y	Y	Y	**Q**	S	G	F	P	N	Q	**R**	E	P	S	Y	S	G	**D**	L	N	Q
GDTS13-F20	Y	Y	Y	**Q**	S	G	F	P	N	Q	**R**	E	P	S	Y	S	G	**N**	L	N	Q
GDTS13-F40	Y	Y	Y	**Q**	S	G	F	P	N	Q	**R**	E	P	S	Y	S	G	**N**	L	N	Q
GDTS13-F60	Y	Y	Y	**Q**	S	G	F	P	N	Q	**K**	E	P	S	Y	S	G	**N**	L	N	Q
GDTS13-F80	Y	Y	Y	**K**	S	G	F	P	N	Q	**K**	E	P	S	Y	S	G	**N**	L	N	Q
GDTS13-F100	Y	Y	Y	**K**	S	G	F	P	N	Q	**K**	E	P	S	Y	S	G	**N**	L	N	Q

Table 2 Virus cross-neutralization testing with GDTS13 and other IBV vaccine strains

Strain	4/91	Massachusetts		Holte	Conn46	GDTS13
		H120	M41			
4/91	64	-	-	-	-	-
H120	-	32	-	-	-	-
M41	-	-	64	-	-	-
Holte	-	-	-	64	-	-
Conn46	-	-	-	-	128	-
GDTS13	0	2	4	4	2	64

Fig. 3 Virus recovery from different tissues after infection with strain GDTS13.
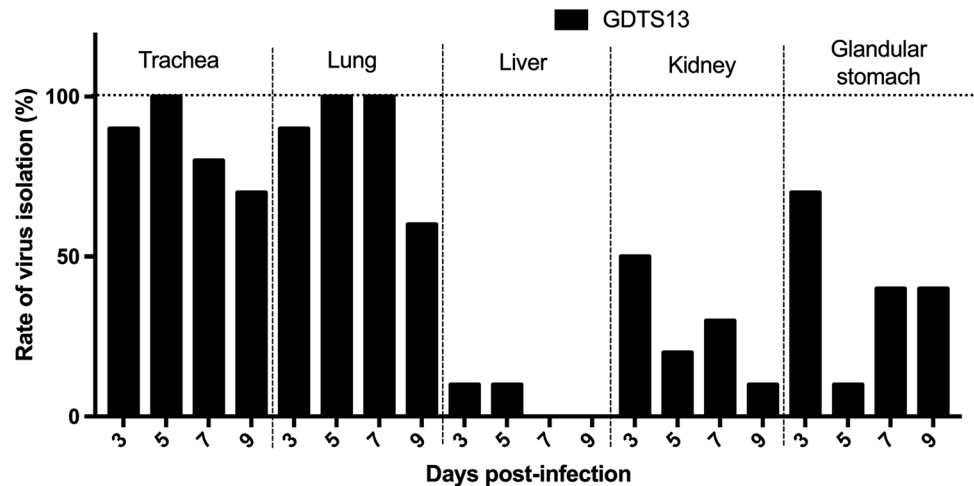
GDTS13-F60 exhibited these signs. No clinical signs were observed in SPF chickens inoculated with GDTS13-F80 or GDTS13-F100 for 15 days after infection.

All chickens were sacrificed and dissected at 15 days postinfection. The percentages of SPF chickens challenged with strains GDTS13-F40 and GDTS13-F60 that showed disease were 60% and 10%, respectively. Macroscopic lesions mainly manifested as tracheal and laryngeal bleeding, increased mucus, bleeding spots in the liver, and, in some chickens, kidney enlargement. No clinical signs or pathological changes were observed in chickens inoculated with GDTS13-F80 or GDTS13-F100.

GDTS13-F100 as a vaccine candidate against IBV in southern China

To evaluate the potential for GDTS13-F100 to serve as an attenuated vaccine, we compared the protective effect of F100 and H120 against challenge with the GDTS13 strain. The morbidity of chickens inoculated with GDTS13 was 90%. In the GDTS13-F100-inoculated groups, no chickens challenged with GDTS13 showed clinical manifestations. However, in the H120-inoculated group, the morbidity of chickens challenged with GDTS13 was 50%. Analysis of oropharyngeal and cloacal swabs showed that all IBV-inoculated chickens in the control groups shed IBV between 3 and 9 days postinfection. No GDTS13-challenged chickens in the GDTS13-F100-immunized group shed virus between 3 and 9 days postinfection, whereas 70% of GDTS13-challenged chickens in the H120-immunized group shed virus between 5 and 9 days postinfection (Fig. 4).
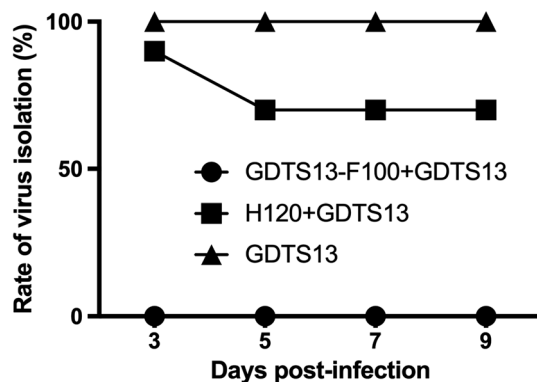

Fig. 4 Virus recovery from oropharyngeal and cloacal swabs from chickens challenged with GDTS13 and vaccine strains of IBV

Discussion

Guangdong province in southern China is considered a reservoir for poultry viruses. This region has a large number of free-range poultry farms and plays a vital role in bird migration, providing ideal conditions for recombination and transmission of avian viruses, including avian influenza virus (AIV) and IBV. Mixed infections of IBV and low-pathogenic AIV can cause severe illness and high mortality, and result in large economic losses for poultry farms [27]. Although vaccines based on the Mass-type and 4/91 strains of IBV have been used for many years in the poultry industry, IB continues to be prevalent in China [15, 28, 29]. Therefore, epidemiological surveillance of avian viruses in Guangdong province is important for protecting the poultry industry in southern China and can provide a theoretical basis for designing a program to prevent and control avian diseases.

IBV is classified into serotypes and genotypes based on the characteristics of the S gene and protein [30]. In this study, we used a new classification scheme to type IBV based on the complete S1 gene sequence [13, 14]. In China, the epidemiology of IBV is complex because of the vast area of virus circulation [15]. Viruses of genotypes GI-7 (4/91-type), GI-13 (TW-type), and GI-19 (QX/LX4-type) have been the ones isolated most frequently in recent years [18, 19], whereas other genotypes are predominant elsewhere in China. For example, from 2011 to 2015, the IBV strains that were dominant in central and eastern China belonged to genotype GI-19, whereas those in southern China were GI-7 [18, 19]. In this study, 16 strains were isolated from 62 tissue samples, showing a positive rate similar to those reported in previous studies [17, 31]. However, in this study, 10 of 16 isolates from Guangdong province in 2016–2017 were classified as genotype GVI-1; only five isolates were classified as GI-19. Our results indicate that genotype GVI-1 IBV is the dominant epidemic strain in Guangdong. Notably, all of the chickens from which isolates were obtained had been immunized with the Mass-type vaccine, but they still displayed clinical signs of IB, suggesting that the Mass-type vaccine used in the poultry industry in China did not effectively prevent infection with genotype GVI-1 IBV strains. Genotype GVI-1 IBV strains have been isolated from chickens in Korea and China since 2007 and were originally classified as 'Korean new cluster II' [22] or 'Chinese new-type' [32] IBVs. Increasingly, however, TC07-2-type genotype GVI strains of IBV have been reported in southern China [18, 33]. The sequences of all GVI-1 strains published in GenBank were downloaded for phylogenetic analysis, and the results indicated that the majority of GVI-1 strains were isolated from southern China, which is consistent

with previous studies [18, 20]. Although there are no data available for GVI-1 in China after 2017, the spread of GVI in southern China should not be ignored. Serotype differences among genetically distinct IBVs generally correlate with variations in the HVRs of the S1 protein gene [11, 34], and differences as low as 5% between S1 sequences of IBV can result in poor cross-protection by vaccines currently in use [15, 35]. Because of the large differences between the S1 protein amino acid sequences of the Mass-type vaccine and GVI-1 strains, infection with GVI-1 IBVs cannot be effectively prevented, and strains of this genotype spread easily through unprotected populations. Therefore, the spread of genotype GVI-1 IBV in southern China urgently requires attention, and more-effective vaccines are needed to limit the circulation of these viruses.

Because of the potential threat posed by GVI-1 IBV to southern China and the lack of a vaccine for protecting chickens against infection with this genotype, we evaluated a newly isolated strain for use as a GVI-1 IBV vaccine. One of these isolates, GDTS13, induced typical signs of IB in 1-day-old SPF chickens, including lethargy, feather ruffling, neck shrinkage, head shaking, sneezing, and diarrhea. Autopsy revealed tracheal, laryngeal, and liver hemorrhage, excessive mucus, and kidney enlargement, and IBV was isolated from the trachea, lungs, kidneys, liver, and glandular stomach. However, the amount of virus in the liver was relatively low, suggesting that this organ is not the major target of strain GDTS13. Furthermore, cross-neutralization testing showed that immune sera against the five vaccine strains (Holte, Conn46, M41, H120, and 4/91) did not effectively neutralize strain GDTS13, indicating that none of the vaccines currently in use can effectively prevent infection with genotype GVI-1 IBV. This may account, at least in part, for the increased incidence of infection with genotype GVI-1 strains in southern China in recent years and explain why genotype GVI-1 was isolated from immunized chickens.

Continuous passage in embryonated SPF chicken eggs is the conventional method for developing attenuated IBV vaccine strains [15, 36], and the pathogenicity of IBV is closely associated with mutations in the S1 protein variants [37, 38]. In this study, the S1 gene of GDTS13-F100 was found to contain four mutations resulting in amino acid changes. Alterations in the S1 protein sequence may be an important reason for the reduced virulence of GDTS13, but this remains to be verified.

According to the OIE standards and requirements of the CVP (2010 Edition) [39], a live vaccine is suitable for use if at least 80% of challenge-vaccinated birds show no evidence of IBV in their trachea, whereas 80% or more of the control birds should have evidence of the presence of the virus. In this study, GDTS13-F100 elicited a more effective immune response (100% protection) than H120 against IBV genotype GVI-1, whereas all chickens in the control group

shed IBV after infection with the GDTS13 strain. Because genotype GVI-1 strains are prevalent in southern China and the widely used Mass-type vaccines cannot protect against them, GDTS13-F100 may be useful as an attenuated vaccine to prevent infection with GVI-1 strains and reduce economic losses due to IB. However, whether the GDTS13 vaccine broadly protects against more-heterologous IBV strains require further analysis.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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