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Serological study reveal different antigenic IBDV strains prevalent in southern China during the years 2000–2017 and also the antigenic differences between the field strains and the commonly used vaccine strains

Xiumiao He[a,b,c,*], Weiwei Wang[b], Guo Chen[b], Pengtao Jiao[b], Zhonghua Ji[b], Lin Yang[a], Ping Wei[b,**]

[a] School of Marine Sciences and Biotechnology/Guangxi Colleges and Universities Key Laboratory of Utilization of Microbial and Botanical Resources, Guangxi University for Nationalities, Nanning, Guangxi 530006, China
[b] Institute for Poultry Science and Health, Guangxi University, Nanning, Guangxi 530004, China
[c] Guangxi Key Laboratory Cultivation Base for Polysaccharide Materials and Modifications, Guangxi University for Nationalities, Nanning, Guangxi 530006, China

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ABSTRACT

The aim of this study was to determine the antigenic relatedness of Infectious Bursal Disease Viruses (IBDVs) in the field in southern China during the period 2000–2017, as well as the antigenic relationship between the field strains and the most commonly used vaccine strains by using a virus neutralization (VN) test *in vitro*. The antigenic relatedness (R) value and the difference in VN titers were analyzed, and the antigenic index based on the sequences of the hypervariable region of VP2 (*v*VP2) of the strains was further evaluated. As a result, the R value of representative field strains showed that there were three subtypes present in the field strains examined, with 7 strains belonging to subtype 1, while strains BH11 and JS7 belonged to subtype 2 and subtype 3, respectively. The commonly used vaccine strains B87 and FW2512 belonged to subtype 1. The analysis of the VN titer differences revealed that all the 136 field strains were classified into subtype 1, except BH11 and JS7. All the field strains in subtype 1 have been divided into at least 5 subgroups, suggesting the antigenic diversity among these strains. The antigenic index based on IBDV-VP2 sequences further confirmed the antigenic differences between the three subtype strains and also the antigenic diversity among the subtype 1. The results demonstrated the antigenic diversity of field IBDVs in southern China during the years 2000–2017 and the antigenic differences between the field strains and the commonly used vaccine strains. This would indicate that the commonly used vaccines are only partially effective. These results enhance our understanding of IBDV genetic evolution and should help to develop more effective vaccines for the control of this disease in the future.

1. Introduction

Infectious Bursal Disease Virus (IBDV) is the etiological agent of Infectious Bursa Disease (IBD), which is an acute, highly contagious and immunosuppressive disease in young chickens and affecting the poultry industry worldwide (Cosgrove, 1962; Müller et al., 2003). There are two serotypes of IBDV that have so far been recognized by the use of monovalent neutralizing antisera: Serotype 1 and Serotype 2. Serotype 1 virus causes clinical disease in chickens and serotype 2 is apathogenic (McFerran et al., 1980). IBDV is a non-enveloped, icosahedral, two-segmented (Segment A and Segment B) double-stranded RNA virus and a member of the *Birnaviridae* family. Segment A encodes the viral structure proteins VP2, VP3 and VP4 and VP5, which have a regulatory function (Ingrao et al., 2013; Eterradossi and Saif, 2013). As the external capsid protein, VP2 elicits a neutralizing antibody (Fahey et al., 1989). Segment B, encoding the viral polymerase VP1, is involved with replication and transcription of the virus, thus playing a role in the virulence of the virus (Coulibaly et al., 2005; Delmas et al., 2004; van den Berg, 2000; Ingrao et al., 2013; Le Nouën et al., 2012; Chen et al., 2018).

IBDV was first reported in 1957 in Gumboro, Delaware, United States (Eterradossi and Saif, 2013), but is currently circulating worldwide. In China, IBD was first reported in Beijing in 1979. Although IBDV is well controlled through implementation of immunization

* Corresponding author at: School of Marine Sciences and Biotechnology/Guangxi Colleges and Universities Key Laboratory of Utilization of Microbial and Botanical Resources, Guangxi University for Nationalities, Nanning, Guangxi 530006, China.
** Corresponding author at: Institute for Poultry Science and Health, Guangxi University, Nanning, Guangxi 530004, China.
E-mail addresses: xiumiaohe0839@sina.com (X. He), pingwei8@126.com (P. Wei).

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practices using attenuated live vaccines, inactivated vaccines and good management, sporadic outbreaks of IBD are still reported (He et al., 2014; Li et al., 2015; Liu et al., 2013). In addition, IBDV reassortment was frequently reported in recent years (Yuwen et al., 2009; He et al., 2014; Abed et al., 2018; Cui et al., 2013; Felice et al., 2017; Jackwood et al., 2016; Lu et al., 2015), leading to the escape of field IBDV strains from the vaccination campaign (He et al., 2014; Chen et al., 2018; Fan et al., 2019), which was of great concern. As an RNA virus, IBDV has a high mutation rate and may thus give rise to viruses with a modified antigenicity. Our group and others have focused on the pathogenicity of these variant strains for many years, and we demonstrated that both the A and B genomic segments of IBDV have contributed to the virulence of IBDV (He et al., 2016). However, the serotype of these recent IBDV field isolates and their antigenic relationship with the commonly used vaccine strains has remained largely unknown in southern China.

In southern China, the farming pattern of local chickens like the well known Three-Yellow chickens is complicated. The biosecurity condition of most chickens farms is inadequate. For example, chickens are free range in open earth fields, disinfection between each brood is incomplete, flocks include multiple-age, and there is non-compliance with an all-in-all-out procedure in practice. IBDV live vaccines are commonly used in broiler and breeding chickens at the age of 12 and 24 days respectively (He et al., 2014; Chen et al., 2018). These situations easily allow IBDV strains with different virulence, antigenicity, and genotypes to co-exist within the farms, resulting in the possibility of reassortment among various IBDVs strains. In our previous studies, the reassortant IBDVs with different pathogenicity and antigenicity in those farms had been identified *in vivo* (He et al., 2014, 2016; Chen et al., 2018). However, we still lack systematic studies and evidence regarding the antigenic relatedness of the field strains since the first report of vvIBDV in Guangxi in 1999. For this purpose, the study was designed to indentify the different antigenicity/subtype of field IBDV strains isolated from Three-Yellow chickens in southern China during the years 2000–2017, as well as to determin their antigenic relationship with the most commonly used vaccine strains. Our results should provide new insights into the antigenicity of dominant IBDV field isolates and could subsequently be helpful to control IBD in the future.

2. Materials and methods

2.1. Viruses

Seven representative IBDV field strains, BH11, NN040124, TSC-2(9), YL052, JS7, TSC-1(3) and HUN0801, used in this study are shown in Table 1. These strains, representing the types of viruses with different partial genome characters, were isolated from chicken farms with outbreaks in different places and years, as we reported previously (He et al., 2014). The commonly used intermediate virulent commercial live vaccine strain B87 as well as intermediate-plus virulent strain FW2512 (HLJ Animal-use Biological Products Co., Ltd., China) were used in the study. The results from our previously phylogenic study showed that the *v*VP2 gene and VP1b gene of both B87 and FW2512 were derived from intermediate IBDV and attenuated IBDV respectively (He et al., 2014). All the other 131 field IBDVs were isolated by our labrotary during the years 2000–2017 from local chicken farms in southern China as we previously discribed (He et al., 2012).

2.2. Cell culture and virus propagation

Chicken-embryo-fibroblast (CEF) cultures were prepared using 9 to 11-day-old embryos from specific-pathogen-free (SPF) chicken eggs (Beijing Merial Vital Laboratory Animal Technology Co., Ltd., Beijing, China). The procedures for harvesting and culturing CEF cells were performed according to those previously described (Jackwood and Saif, 1987; Rosenberger et al., 1975). Cells were incubated at 37 °C in a humidified 5% CO2 atmosphere. These cells were used to propagate viruses.

The procedure for propagation of the virus on CEF cells were previously described by our group (Yang et al., 2007) and the titration of the virus on CEF cells were similar to those we previously described on *Vero* cells (He et al., 2016). Briefly, all the field isolates were propagated in CEF cells for 1–2 passages until the cytopathic effect (CPE) was observed, and then virus plague-purified were performed in CEF cells and the purified virus were titrated for further VN tests. Before titration, the sequences of the *v*VP2 of the cell adapted strains were amplified and analyzed according to He et al. (2012), only those without mutations in the *v*VP2 amino acid as compared to the original strains could be used for further VN tests. The viruses were titrated by inoculating 10-fold serial dilutions (10^{-1}–10^{-10}) into the CEF cells. The number value for the cytopathic effect (CPE) was recorded and the titrations were calculated as 50% tissue culture infective dose ($TCID_{50}$) using the Reed-Muench method (Reed and Muench, 1938).

2.3. Hyperimmune sera

Antigen for hyperimmune sera preparation was described in our previously published paper (Chen et al., 2018). Briefly, the harvested viruses were inactivated using 0.5% formaldehyde with continuous stirring at 37 °C for 24 h. The inactivation efficiency was performed by 3 blind passages of the inactivated virus in 5 SPF embryonated chicken eggs. After complete inactivation, the inactivated viruses with 4% Tween 80 were added to form an aqueous phase. The No. 10 mineral oil was added with Span 80 in a ratio of 10/1 to create the oil phase. The aqueous phase was emulsified with the oil phase in a ratio of 40/60 (V/V) and the antigens were stored at 4 °C.

Rabbit hyperimmune monovalent sera against each IBDV strain were prepared as described (Jackwood et al., 1982). Rabbits were inoculated intramuscularly and subcutaneously 3 times at 10 days intervals. The inocula contained approximately 10^4 to 10^6 $TCID_{50}$ of each virus. No antibody to IBDV was detected in rabbit sera before inoculation. After 10 days past the third inoculation, the sera were tested with IBDVs by the agar gel precipitation test (AGP) test. The rabbit serum with AGP titer of 1:32 and higher was harvested and heat inactivated at 56 °C for 30 min before use in the following cross

Table 1
The representative natural reassortant IBDV strains used in the study.

Strain	Year	Location	Immune status of the flock	vVP2 (Genotype) (He et al., 2014)	VP1b (Genotype) (He et al., 2014)
FW2512	2004	Commercial vaccine strain		C2-intermediate IBDV	Attenuated IBDV
B87	2004	Commercial vaccine strain		C2-intermediate IBDV	Attenuated IBDV
BH11	2004	Beihai/Guangxi	Yes	C1-vvIBDV	Attenuated IBDV
NN040124	2004	Nanning/Guangxi	Yes	C2-intermediate IBDV	Attenuated IBDV
TSC-2 (9)	2005	Yulin/Guangxi	Yes	G2-vvIBDV	Attenuated IBDV
YL052	2005	Yulin /Guangxi	Yes	C2-intermediate IBDV	cIBDV (002-73)
JS7	2005	Binhai/Jiangsu	Yes	G2-vvIBDV	cIBDV (002-73)
TSC-1 (3)	2005	Yulin /Guangxi	Yes	C3-attenuated IBDV	Attenuated IBDV
HUN0801	2008	Hunan	Yes	G1-vvIBDV	Attenuated IBDV

Table 2
Antigenic relatedness of vaccines and representative field strains of IBDV.

	Fw2512	B87	YL052	BH11	Tsc-2 (9)	NN040124	JS7	HUN0801	Tsc-1 (3)
Fw2512	1								
B87	0.83	1							
YL052	0.84	0.95	1						
BH11	0.21	0.18	0.32	1					
Tsc-2(9)	0.82	1	0.96	0.15	1				
NN040124	1	0.82	0.94	0.23	0.90	1			
JS7	0.46	0.45	0.44	0.12	0.56	0.43	1		
HUN0801	0.80	0.86	0.82	0.14	0.87	0.80	0.42	1	
Tsc-1 (3)	0.81	0.87	0.91	0.19	0.81	0.91	0.36	0.92	1

Numbers in the table are relatedness values (R) calculated according to Archetti and Horsfall (1950). A homologous R value is considered 1.00; $R \geq 0.8$ is considered to be the same subtype; $R < 0.1$ is considered to be a different serotype. Notes BH11 is one subtype, e.g subtype 2, *Js*7 is one subtype, e.g subtype 3, and the other isolates including the vaccine strains are the same subtype, e.g subtype 1. All viruses are serotype I.

neutralization or virus-neutralization (VN) tests.

2.4. Experiment design

A cross VN test was performed to analyze the 9 IBDV strains, including 7 representative field isolates and the 2 commercial vaccine strains. After determining the subtypes of these strains, the representative subtypes with the corresponding anti-IBDV serum were selected and used for the VN tests between the field IBDV strains isolated during the period 2000–2017 from southern China and the representative anti-IBDV serum. The VN titer of each isolate was recorded, and the VN titer difference was analyzed further and indicated as: VN titer difference = VN1-VN2, in which the VN1 and VN2 represent the VN titer of field strains and the homologous strains, respectively. Finally, the antigenic related gene of IBDV, which is the hypervariable gene of VP2 (*v*VP2) of the representative field strains were analyzed for the viral antigenic index.

2.5. Virus-neutralization test

A virus-neutralization (VN) test in CEF was conducted as previously described by Jackwood group (Jackwood et al., 1982; Jackwood and Saif, 1987). Antibody titers to homologous and heterologous IBDV strains were determined using the constant-virus varying-antibody technique.

2.6. Antigenic relatedness

The following criterion was used to classify viruses into serotypes according to the cross virus-neutralization (VN) titers: antisera containing 20 units of antibody to one IBDV strain that did not neutralize 100 $TCID_{50}$ of virus belong to another IBDV serotype. An antibody unit was defined as the lowest concentration of antiserum that neutralized 100 $TCID_{50}$ of homologous virus. To facilitate the interpretation of cross VN test results, the following formula of Archetti and Horsfall (1950) was employed to express the antigenic relatedness of viruses within a serotype: $R = \sqrt{r1 \times r2}$, in which the ratio $r1$ is determined by dividing the heterologous sub-serotype titer obtained with virus 2 by the homologous titer obtained with virus 1, and the ratio $r2$ is determined by dividing the heterologous titer obtained with virus 1 by the homologous titer obtained with virus 2. The formula yields an antibody titer ratio (R) that expresses the antigenic relatedness between two viruses when both antigens and antisera are used in a cross-neutralization test. R values were calculated from a geometric mean antibody titer obtained from a minimum of three tests. The R value equal or close to 1 indicates antigenic similarity between two virus strains. R value between 0 and 0.10 = a serotype difference; 0.11–0.32 = a major subtype difference; 0.33–0.70 = a minor subtype difference; > 0.70 = little or no difference (Brooksby, 1967). To establish relationships between the strains, a hierarchical cluster analysis was further carried out with the R values of the strains by using the Statistical Package for Social Sciences (SPSS) program.

2.7. Antigenic index analysis

The nucleotides of the *v*VP2 of the representative subtype strains were downloaded from NCBI and were described in our previous study (He et al., 2012, 2014). The antigenic index was calculated using the Jameson–Wolf algorithm (Jameson and Wolf, 1988) in the software DNASTAR Lasergene version 7.2. (DNASTAR Inc., Madison, WI, USA) to predict the differential antigenic determinants which existed among the field isolates. The GenBank accession numbers for *v*VP2 used in analysis were: B87 (D49706), GD10111 (JQ260877), NN1005 (JQ260881), NN1172 (JQ398618), NN040124 (DQ656502), YL052 (DQ656521), BH11 (656497) and *JS*7 (DQ656501).

3. Results

3.1. Cross neutralization tests revealed that there were three subtypes of IBDVs prevalent in southern China

In order to understand the current status of antigenicity of IBDV prevalent in southern China, 7 representative field strains were selected according to the phylogenetic analysis based on both *v*VP2 and VP1-b as we described previously (He et al., 2014). The results of cross VN tests, as expressed as relatedness (R) values, were shown in Table 2. The R values of isolate BH11 to all the other strains ranged between 0.12–0.32, indicating a major subtype difference. Next, the R value between isolate JS7 and BH11 was 0.12 (major subtype difference). The R values of *JS*7 with the other remaining strains, including two commonly used vaccine strains, ranged between 0.36–0.56 (minor subtype difference). Finally, The R values of the remaining 5 representative field strains and 2 commonly used vaccine strains varied from 0.81–0.96, which were higher than 0.7 and that meant there was little or no difference between these strains. None of the R values were lower than 0.1, indicating that all the representative field strains and the commonly used vaccine strains were classified into the same serotype.

In order to further determine the relationships between the representative strains, a hierarchical cluster analysis was carried out with the R values of these strains by using the SPSS program. As shown in Fig. 1., the BH11 belonged to one cluster, JS7 belonged to another seperate cluster, while the two commonly used vaccine strains and the remaining 5 representative field strains NN040124, HuN0801, TSC-1(3), TSC-2(9), YL052 were grouped to a third seperate big cluster. These results were consistent with the R value analysis. Given that viruses belonging to the same cluster or group were more closely related, we concluded from the results of the cluster analysis and the R values (Table 2) that the 7 representative field IBDVs, and the vaccine strains B87 and FW2512 can be grouped into three subtypes. The field strains NN040124, HuN0801, TSC-1(3), TSC-2(9) and YL052 were

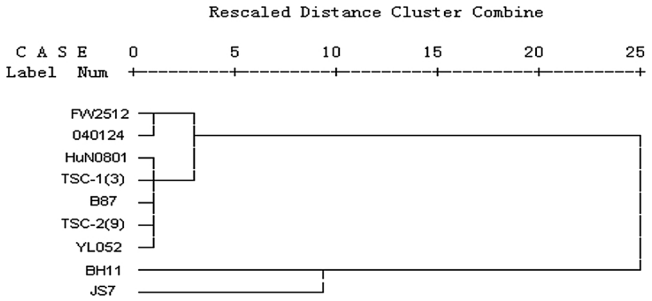

Fig. 1. Phylogenetic analysis of subtypes of representative IBDV field isolates. The phylogenetic tree is based on the relatedness values (R) from Table 2. Consistent with the R value, BH11 is grouped to one clade; JS7 is grouped to another one clade; and the left isolates and vaccine strains were grouped to a big clade, but are definitely divided into two subgroups.

classified into subtype 1, while BH11 belonged to subtype 2 and JS7 belonged to subtype 3. The IBDV strains in the subtype 1 were divided into two subgroups, one subgroup included vaccine strain FW2512 and field strain NN040124, another subgroup included vaccine strain B87 and the field strains HuN0801, TSC-1(3), TSC-2(9) and YL052.

In order to comprehensively understand the antigenic difference between all the IBDV field strains and the three subtypes identified, the representative hyperimmune sera of different subtypes were selected, *e.g.*, anti-BH11, anti-JS7, anti-NN040124, anti-TSC-1(3) and anti-HuN0801 sera. VN tests were carried out between the field strains and the representative sera, and the VN titer difference was analyzed. In order to make conclusive results depending only on the VN titer difference, the criterion was established first as shown in Table 3. As indicated, the VN titer differences among subtype 1 strains varied from 10^{0} to $10^{0.2}$, while the difference between subtype 1 and 2 (BH11) was $10^{0.5}$ to $10^{0.83}$, and those between subtype 1 and 3 was $10^{0.33}$ to $10^{0.53}$, subtype 2 and 3 was $10^{1.11}$. We thus established that if the VN titer difference between field isolates and all the homologous representative subtype 1 viruses were lower than $10^{0.5}$, then we could conclude that those strains belong to subtype 1, and the same criteria for subtype 2 and 3. If the VN titer differences were higher than all the reference subtype strains ($> 10^{0.5}$), then that strain would be hypothesized as a new subtype. Based on this criterion, 130 field IBDVs showed a VN titer difference close to subtype 1 that varied from 10^{0}-$10^{0.5}$ (Table 4), and thus belonged to subtype 1. Only isolate NN1172 showed a VN titer difference between $10^{0.67}$ to $10^{1.525}$ to all the subtype 1 anti-sera; $10^{2.15}$ to subtype 2; and $10^{1.855}$ to subtype 3, which might suggest a new subtype. However, when a hierarchical cluster analysis was performed based on the VN titer differences and the R values, as shown in Fig. 2A (isolates obtained before 2010) and Fig. 2B (isolates obtained after 2010), all the 131 field isolates detected, including NN1172, were grouped with subtype 1 virus. Interestingly, all the isolates grouped with subtype 1 virus in Fig. 2A and B were all divided into 5 small subgroups. These results suggested that most isolates were antigenically similar to subtype 1 IBDV. However, there were minor antigenic differences among subtype 1 strains.

Table 3
The neutralization titer difference among different subtype IBDV strains.

Strains	Hyperimmune rabbit Serum		
	Subtype 1	Subtype 2	Subtype 3
Subtype 1	$10^{0.0-0.2}$		
Subtype 2	$10^{0.5-0.83}$	$10^{0.0}$	
Subtype 3	$10^{0.33-0.53}$	$10^{1.11}$	$10^{0.0}$

Numbers in the table are VN titer differences between representative IBDV strains and the specific hyperimmune rabbit Serum. Note that the VN titer differences among the subtype 1 strains varied from 10^{0} to $10^{0.2}$, while the difference between subtype 1 and 2 (BH11) ranged from $10^{0.5}$ to $10^{0.83}$, and those between subtype 1 and 3 from $10^{0.33}$ to $10^{0.53}$, subtype 2 and 3 was $10^{1.11}$.

3.2. The commonly used vaccine strains were antigenically different from the field isolates

The vaccine strains, FW2512 and B87, which were commonly used in China were grouped with the subtype 1 virus as shown in Fig. 1. However, all strains in the subtype 1 group were divided into two small groups: one included FW2512 and 040124, the other one included B87, HuN0801, Tsc-1(3) and YL052. These groups were completely different from the other two field strains, BH11 and JS7, suggesting major antigenic differences between the two commonly used vaccine strains and the field strains. In order to further explore the antigenic differences between field IBDV strains and the vaccine strains, the VN titer differences between the field strains and vaccine strains FW2512 or B87 were further determined, and the results are shown in Table 5. There were 43.2% of the isolates that showed a difference of 10^{0-1} in VN titer to FW2512, while 64.5% of the isolates showed a difference of 10^{0-1} to B87. Also, 23.5% and 16.7% of the isolates showed a difference of $10^{1.1}$–10^{2} to FW2512 and B87, respectively. Finally, 33.3% and 18.8% (26/138) of the isolates showed a difference of $10^{2.1}$–10^{3} to FW2512 and B87, respectively. Among these isolates, only 18.5% of the isolates showed a difference of 10^{0}–10^{1} in VN titer to both FW2512 and B87. Also, 4.94% (4/81) of the isolates showed a difference of $10^{1.1}$–10^{2} in VN titer to FW2512 and B87, and there were 25.9% (21/81) of the isolates which showed a difference of $10^{2.1}$–10^{3} in VN titer to both FW2512 and B87. These results indicated that most of the isolates were antigenically different from FW2512 and B87, suggesting that the vaccines could not provide 100% protection to the field IBDV infection.

3.3. The analysis of IBDV-VP2 gene revealed the differential antigenic index among the field IBDV strains and the vaccine strains

The VP2 gene is known to be the determinant of the antigenicity of IBDV. In order to determine if the differential antigenicity among field isolates and vaccine strains was correlated to the epitopes in VP2 peptide, the *v*VP2 sequences of some of the IBDV field strains from subtype 1, the commonly used vaccine strain B87, subtype 2 and subtype 3 strains in the NCBI database were downloaded and analyzed by DNAStar. The antigenic index plots were predicted using the Jameson–Wolf algorithm. As shown in Fig. 3, the antigenic sites in the regions of 212–224 and 279–290 of the amino-acid (aa) residues were different between the three subtype strains. Even between the subtype 1 strains, 151120 and NN1172, there is weak antigenicity in the region of 212–224 aa residues, while other strains, including BH11 and JS7, showed strong antigenicity in this region. In the region of 279–290 aa residues, there were two separate antigenic regions, 279–283 and 283–290. In the region of 279–283 aa residues, NN040124, YL052, BH11 showed a strong antigenic signal, while 151120, GD10111, NN1005, NN1172 and JS7 showed weak antigenicity, and the vaccine strain B87 showed the weakest signal of antigenicity in this region. In the region of 283–290 aa residues, B87 showed the strongest signal, followed by strains of 151120, GD10111, NN1005, NN1172 and JS7. However, NN040124, YL052 and BH11 showed no signal in this region. These results suggested that the antigenic index of the VP2 protein of the field IBDVs and the vaccine strains were different.

4. Discussion

The antigenic diversity of IBDVs existing in the field has been confirmed for many years. As early as 1980s, McFerran et al. (1980) alluded to antigenic differences among the serotype I IBDVs, some isolates showing only a 30% cross reaction with the vaccine strain. Saif (1984) reported an isolate of serotype I obtained from bursas of 7-day-

Table 4
The VN titer difference between some field IBDVs and the representative subtype strains.

Isolates	Place/year	HUN0801	NN040124	TSC-1(3)	B87	BH11	JS7	Isolates	Place/year	HUN0801	NN040124	TSC-1(3)	B87	BH11	JS7
WM1001	Guangxi/2010	$10^{0.35}$	$10^{0.22}$	$10^{1.02}$	$10^{0.92}$	$10^{1.35}$	$10^{2.18}$	150312	Guangxi/2015	$10^{0.1}$	$10^{0.02}$	$10^{0.02}$	$10^{0.15}$	$10^{0.59}$	$10^{1.16}$
HP1001	Guangxi/2010	$10^{0.9}$	$10^{0.2}$	$10^{0.259}$	$10^{0.82}$	$10^{1.39}$	$10^{1.725}$	150315	Guangxi/2015	$10^{0.42}$	$10^{0.21}$	$10^{0.22}$	$10^{0.16}$	$10^{1.75}$	$10^{1.85}$
GL1001	Guangxi/2010	$10^{0.495}$	$10^{0.74}$	$10^{0.3}$	$10^{0.82}$	$10^{1.185}$	$10^{1.665}$	150706	Guangxi/2015	$10^{0.7}$	$10^{0.34}$	$10^{0.45}$	$10^{0.55}$	$10^{2.37}$	$10^{2.66}$
WM1004	Guangxi/2010	$10^{0.35}$	$10^{0.06}$	$10^{0.037}$	$10^{0.14}$	$10^{1.249}$	$10^{1.426}$	150813	Guangxi/2015	$10^{0.06}$	$10^{0.15}$	$10^{0.35}$	$10^{0.07}$	$10^{1.39}$	$10^{1.87}$
NN1005	Guangxi/2010	$10^{0.377}$	$10^{0.405}$	$10^{0.165}$	$10^{0.14}$	$10^{1.145}$	$10^{1.34}$	151120	Guangxi/2015	$10^{0.03}$	$10^{0.05}$	$10^{0.27}$	10^{0}	$10^{0.5}$	$10^{1.08}$
GD10114	Guangdong/2010	$10^{0.31}$	$10^{0.32}$	$10^{0.152}$	$10^{0.02}$	$10^{1.15}$	$10^{1.33}$	GY160228	Guangxi/2016	$10^{0.22}$	$10^{0.44}$	$10^{0.38}$	$10^{0.5}$	$10^{1.01}$	$10^{1.45}$
YL10111	Guangxi/2010	$10^{0.4}$	$10^{0.17}$	10^{0}	$10^{0.22}$	$10^{1.245}$	$10^{1.139}$	YL160304	Guangxi/2016	$10^{0.02}$	$10^{0.14}$	$10^{0.13}$	$10^{0.04}$	$10^{0.63}$	$10^{1.05}$
YL10112	Guangxi/2010	$10^{0.35}$	$10^{0.295}$	$10^{0.25}$	$10^{0.17}$	$10^{1.35}$	$10^{1.229}$	NN160401	Guangxi/2016	$10^{0.42}$	$10^{0.2}$	$10^{0.02}$	$10^{0.14}$	$10^{0.79}$	$10^{1.43}$
NN1163	Guangxi/2011	$10^{0.35}$	$10^{0.72}$	$10^{1.25}$	$10^{1.17}$	$10^{3.3}$	$10^{1.715}$	YL160304	Guangxi/2016	$10^{0.02}$	$10^{0.14}$	$10^{0.13}$	$10^{0.04}$	$10^{0.63}$	$10^{1.05}$
YN1161	Yunnan/2011	$10^{0.85}$	$10^{0.57}$	$10^{1.45}$	$10^{1.12}$	10^{2}	$10^{2.485}$	NN160401	Guangxi/2016	$10^{0.42}$	$10^{0.2}$	$10^{0.02}$	$10^{0.14}$	$10^{0.79}$	$10^{1.43}$
NN1172	Guangxi/2011	10^{1}	$10^{1.17}$	$10^{1.525}$	$10^{0.67}$	$10^{2.15}$	$10^{1.855}$	NN160510	Guangxi/2016	$10^{0.14}$	$10^{0.04}$	$10^{0.04}$	$10^{0.42}$	$10^{0.62}$	$10^{0.51}$
LS1107	Zhejiang/2011	$10^{0.35}$	$10^{0.02}$	$10^{0.14}$	$10^{0.06}$	$10^{0.96}$	$10^{0.68}$	YL160810	Guangxi/2016	$10^{0.38}$	$10^{0.05}$	$10^{0.15}$	$10^{0.47}$	$10^{0.85}$	10^{1}
YY1195	Zhejiang/2011	$10^{0.28}$	$10^{0.23}$	$10^{0.35}$	$10^{0.14}$	$10^{1.25}$	$10^{1.77}$	NN160906	Guangxi/2016	$10^{0.42}$	$10^{0.44}$	$10^{0.3}$	$10^{0.42}$	$10^{1.22}$	$10^{1.25}$
YY1213	Zhejiang/2012	$10^{0.58}$	$10^{0.35}$	$10^{0.5}$	$10^{0.22}$	$10^{1.98}$	$10^{1.76}$	LB161029	Guangxi/2016	$10^{0.21}$	$10^{0.2}$	$10^{0.04}$	$10^{0.18}$	$10^{1.17}$	$10^{0.97}$
NN1207	Guangxi/2012	$10^{0.42}$	$10^{0.43}$	$10^{0.49}$	$10^{0.14}$	$10^{0.05}$	$10^{1.77}$	NN1611233	Guangxi/2016	$10^{0.31}$	$10^{0.04}$	$10^{0.12}$	$10^{0.13}$	$10^{0.88}$	$10^{1.35}$
NB1109	Zhejiang/2011	$10^{0.72}$	$10^{0.66}$	$10^{0.52}$	$10^{0.34}$	$10^{1.72}$	$10^{1.67}$	YL170114	Guangxi/2017	$10^{0.51}$	$10^{0.46}$	$10^{0.04}$	$10^{0.23}$	$10^{2.25}$	$10^{1.33}$
WM12061	Guangxi/2012	$10^{0.7}$	$10^{0.44}$	$10^{0.39}$	$10^{0.48}$	$10^{2.15}$	$10^{1.56}$	BL170125	Guangxi/2017	$10^{0.29}$	$10^{0.33}$	$10^{0.19}$	$10^{0.45}$	$10^{1.98}$	10^{2}
140007	Guangxi/2014	$10^{0.14}$	$10^{0.34}$	$10^{0.25}$	$10^{0.02}$	$10^{1.17}$	$10^{1.14}$	YL170220	Guangxi/2017	$10^{0.7}$	$10^{0.53}$	$10^{0.37}$	$10^{0.34}$	$10^{1.23}$	$10^{1.14}$
140021	Guangxi/2014	$10^{0.43}$	$10^{0.04}$	$10^{0.03}$	$10^{0.23}$	$10^{2.15}$	$10^{2.43}$	NN170307	Guangxi/2017	$10^{0.61}$	$10^{0.11}$	$10^{0.04}$	$10^{0.06}$	$10^{1.28}$	$10^{1.23}$
140028	Guangxi/2014	$10^{0.58}$	$10^{0.55}$	$10^{0.37}$	$10^{0.32}$	$10^{1.25}$	$10^{1.13}$	LZ170322	Guangxi/2017	$10^{0.4}$	$10^{0.75}$	$10^{0.76}$	$10^{1.01}$	$10^{1.25}$	$10^{1.13}$
140045	Guangxi/2014	$10^{0.42}$	$10^{0.3}$	$10^{0.05}$	$10^{0.44}$	$10^{1.49}$	$10^{1.22}$	NN170502	Guangxi/2017	$10^{0.49}$	$10^{0.87}$	$10^{1.22}$	$10^{0.8}$	$10^{1.15}$	$10^{1.44}$
140811	Guangxi/2014	$10^{0.18}$	$10^{0.44}$	$10^{0.08}$	$10^{0.05}$	$10^{1.26}$	$10^{1.27}$	YL170801	Guangxi/2017	$10^{0.4}$	$10^{0.45}$	$10^{0.24}$	$10^{0.12}$	$10^{1.59}$	$10^{1.16}$

The VN titer differences are expressed as the VN titer of the field strains with the representative anti-serum – VN titer of the homologous strains and the anti-serum.

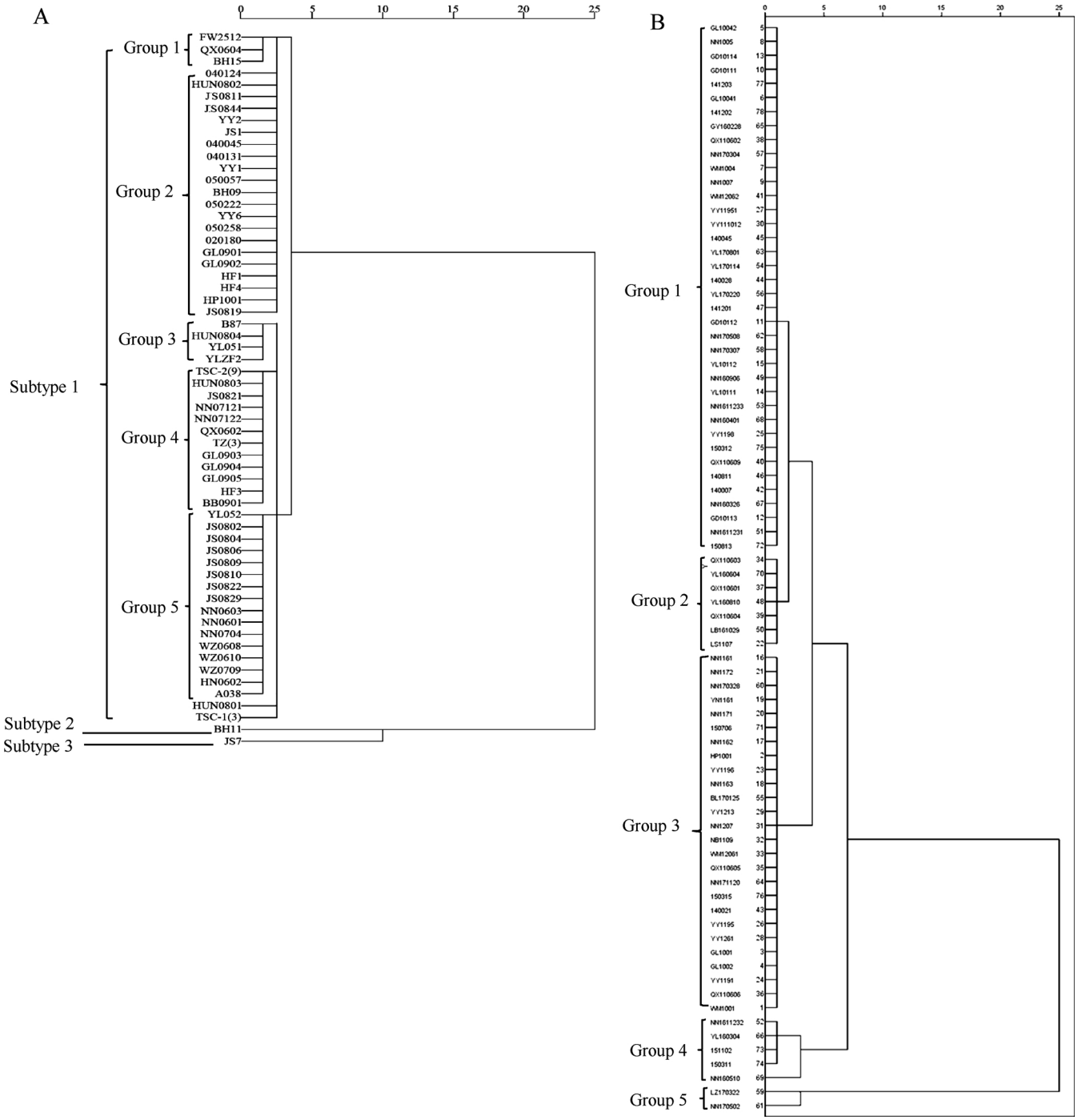

Fig. 2. Hierarchical cluster analysis of the subtype 1 IBDV field strains. The isolates prior to the year 2010 were analyzed, subtype 2 strain BH11 and subtype 3 strain JS7 were used as control. Consistent with the results from R value, BH11 and JS7 were divided into two clades, and the other field isolates and commonly used vaccine strains grouped to a big clade. (A). The isolates after the year 2010 in subtype 1 were further analyzed based on the VN titer difference (B). Note that the isolates were divided into 5 small groups in both Fig A and B.

old broilers in Maryland that had high levels of maternal antibody to serotype I IBDV and the VN tests indicated that the isolate was antigenically distinct from several vaccine and field strains of IBDV. Rosenberger and Cloud (1985) isolated four serotype I IBDVs that also differed antigenically from reference strains. Jackwood and Saif (1987) confirmed by cross-neutralization tests that there were six subtypes among the 13 serotype I strains tested, including eight serotype I commercial vaccine strains and five serotype I field strains. Recent reports described the isolation of serotype I IBDV in commercial broilers that may cause disease in vaccinated flocks. Domanska group (Domanska et al., 2004) confirmed in an Antigen-Capture-ELISA with a panel of neutralizing monoclonal antibodies (Mabs) that all isolates during 1993–2000 proved antigenically and genetically related to a typical vvIBDV strain, excepting the Polish isolate 93/35 which proved related to the antigenically modified vvIBDV strain 91168, although no epidemiological relationship had been documented between these viruses in the field. In 2018, Fan et al. (2019) identified novel variant IBDVs for the first time in eastern China, which were obviously different from the American IBDV variants at the molecular level.

In southern China, we have reported the challenging farming

Table 5
The percentage of isolates based on the neutralization titer difference between field isolates and vaccine strains.

Strain	Difference in VN titers		
	10^{0-1}	$10^{1.1-2}$	$10^{2.1-3}$
FW2512	43.2%(35/81)	23.5%(19/81)	33.3% (27/81)
B87	64.5% (89/138)	16.7%(23/138)	18.8%(26/138)
FW2512, B87	18.5%(15/81)	4.94%(4/81)	25.9%(21/81)

Neutralization tests were performed between IBDV field strains and anti-FW2512 serum or anti-B87serum, respectively. The VN titer difference between VN titers of field strains with anti-FW2512 serum or anti-B87 serum and VN titer of the vaccine homologous strains were calculated. Numbers in the table are the percentage of the isolates among the field isolates tested with the anti-FW2512 serum or/and anti- B87 serum in a certain range of VN titer difference.

situations in the local free-range farms (Chen et al., 2018). IBDV field isolates from those farms after the year of 2000 are mostly reassortant viruses based on the representative fragments of both VP1 and VP2, and that 94% of them were isolated from vaccinated flocks (He et al., 2012, 2014). We also confirmed that the pathogenicity and the antigenicity of 4 representative strains have changed (He et al., 2016, Chen et al., 2018). In the present study, we confirmed that antigenic diversity existed among the serotype 1 strains in southern China during the years 2000–2017. We found that there were at least three subtypes of IBDV that existed. Further, even in the same subtype, there were still minor differences existing between the strains as demonstrated by the hierarchical cluster analysis based on the VN titer and the R values as shown in Figs. 1 and 2. To our knowledge, this is the first report of antigenic diversity of IBDVs existing in the field since 2000 in southern China, based on the analysis of 138 isolates. The result was consistent with our previous result, which was performed *in vivo* and revealed that different antigenic subtypes co-existed among the field IBDV strains (Chen et al., 2018). Zhou et al. (1999) also confirmed that different subtypes of IBDV existed in the isolates from earlier times in Zhejiang and Sichuan provinces in China. We reported similar results based on an analysis of Guangxi field isolates (Huang et al., 2009). In conclusion, all these results suggest that the co-existence of different antigenic subtypes of IBDV strains in the field could be one of the important reasons for the constant, sporadic outbreaks of IBDV in these areas. The predominant farming situation in southern China, which is characterized by free-range in natural earth fields with considerably inadequate biosecurity conditions, easily allowed these kinds of strains to co-exist and has highlighted the necessity to find better ways to control IBD.

B87, which is a major live vaccine strain widely used in China for IBDV control (China Veterinary Pharmacopoeia Commission, 2010), had been shown in our previous study to only provide 60–80% protection against four representative field IBDV strains (Chen et al., 2018). In this study, at least 35.5% of field isolates showed antigenic difference to B87, at least 30.8% of field isolates showed antigenic difference to both B87 and FW2512, consistent with the result from hierarchical cluster analysis (Fig. 2). Other studies (McFerran et al., 1980; Saif, 1984; Jackwood and Saif, 1987; Zhou et al., 1999) also reported that field IBDV isolates were antigenically different from commonly used vaccine strains. In southern China, IBDV live vaccines are commonly used in broiler and breeding chickens at the age of 12 and 24 days (He et al., 2014). In the field, the program of one vaccination with the OEV in the breeder flock before laying, and one vaccination in the chicks with the attenuated live vaccine strain B87 during 7–14 days of age or the IBDV-VP2 recombinant HVT live vaccine at

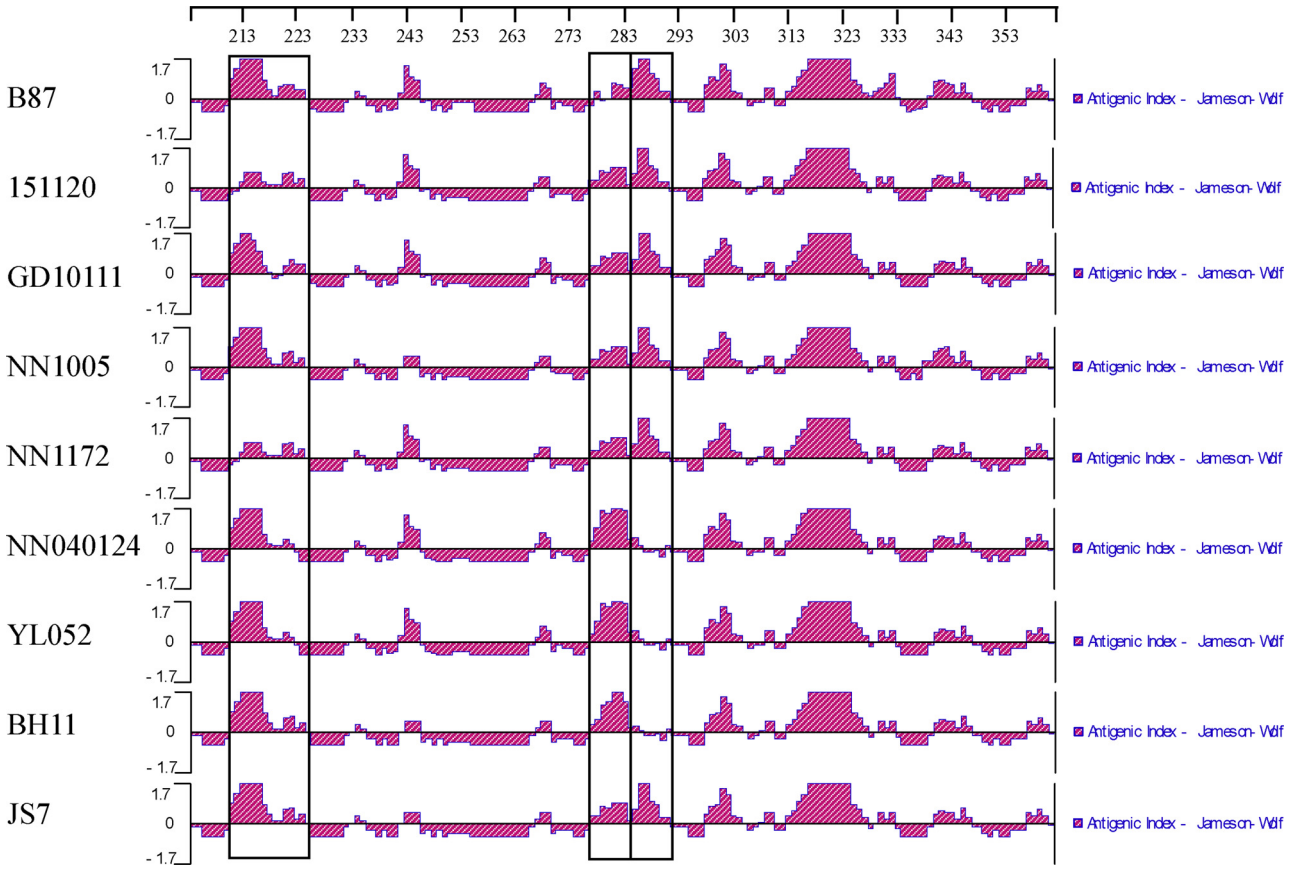

Fig. 3. Antigenic index plots were generated from the amino acid sequence of the vVP2 of the IBDV strains by using the Jameson–Wolf algorithm. The hatched lines above the plots refer to the amino acid numbers of the protein sequence. Graphic increased positivity shows predictive antigenic sites. Alignment shows that predicted antigenic sites were different between different subtypes, and commonly used vaccine strain B87 and field strains as indicated by Black boxes.

hatching (Le Gros et al., 2009) was in practice. However, the IBD outbreaks and immunosuppression from IBD were still commonly reported in the free-range system in southern China (He et al., 2014; Li et al., 2015; Liu et al., 2013). Studies from Michel and Jackwood (2017) and Durairaj et al. (2011) suggested that antigenic drift might occur in some field strains in response to antigenic pressure from vaccination. Consistent with those studies, our cross VN tests and R value analysis confirmed that the field strains antigenically changed. Taken together, the antigenic differences between field strains and vaccine strains revealed from this study based on VN titer and R value further confirmed our previous speculation (Chen et al., 2018) that with the pressure of frequent vaccination using the B87 strain or other live vaccines, the field viruses have evolved and have undergone antigenic drift, resulting in a decrease of the protection provided by commonly used live vaccines. This probably is one of the reasons why IBD outbreaks still exist in the vaccinated flocks and why there remains a complicated IBD epidemic pattern in free-range chickens in southern China.

The antigenic index offers a means of evaluating potential regions of antigenicity directly from the primary sequence of a protein (Jameson and Wolf, 1988). VP2 is the only known IBDV antigen capable of inducing neutralizing antibodies in chickens, and the four hydrophilic regions (peak A: aa residues 210–225, minor peak 1: 247–254, minor peak 2: 281–292, peak B: 312–324) in the *v*VP2 have been found to influence the antigenicity and the MAb reactivity pattern of IBDV (Boot et al., 2000; Jackwood and Sommer-Wagner, 2011; Letzel et al., 2007; Vakharia et al., 1994). Interestingly, consistent with the VN tests results, we found that the antigenic index of the isolates tested based on *v*VP2 was different among the field isolates tested which also suggested antigenic differences and antigenic diversity existed in the field based on the molecular level. However, we couldn't find any logical or specific changes of the antigenic index plot in the four hydrophilic regions of the isolates related to the subtype or sub-group among the subtype 1 strains. For example, the antigenic index of BH11 (subtype 2) and YL052 (subtype 1), JS7 (subtype 3) and NN1005 (subtype 1) were similar (Fig. 3). The Durairaj group (Durairaj et al., 2011) revealed that amino acid exchanges within a certain region of the VP2 molecule resulted in differences in the antigenicity of the virus. Molecular epidemiology studies from our group and others (Chen et al., 2018; He et al., 2014; Fan et al., 2019; Islam et al., 2012) also implied that both genome segments A and B of IBDV might be contributed to the antigenicity and the pathogenicity of IBDV. Altogether, we speculated that other than VP2 or the four hydrophilic regions in the *v*VP2, there might be other viral gene/genes affects/affect the antigenicity of the virus. However, the exact relationship between the distinct genetic characters and the subtype of the field isolates need further investigation.

In this study, hyperimmune sera were prepared by using formaldehyde-inactivated IBDVs. It is reported that formaldehyde might alter the viral-neutralizing epitopes during the process of inactivation, resulting in a poor neutralizing antibody response (Fan et al., 2015; Openshaw et al., 2001). However, other researches reported that the impaired impact of formaldehyde on viral antigenic epitope in inactivation was different between various viruses (review from Delrue et al., 2012). In the case of Ross River virus (Kistner et al., 2007), formaldehyde inactivation wouldn't modify viral protein, however, viral protein modification was found in PRRSV (Delrue et al., 2009) and HIV (Rossio et al., 1998). In the case of IBDV, the anti-serum derived from formaldehyde-inactivated IBDVs could completely neutralize their homologous viruses in most cases. Our group and others (Chen et al., 2018; Hoshi et al., 1995) also found that formaldehyde inactivated IBD vaccine could provide full protection in chickens when challenged with homologous virus, which suggested that, indeed, formaldehyde inactivation did not have significant effect on altering of IBDV epitopes. Nevertheless, the exact mechanism of the effect induced by formaldehyde on IBDV epitopes might need further investigation in the future.

In conclusion, for the first time, the antigenicity of the field IBDV isolates from the majority of chicken farms in Southern China during the period 2000–2017 were determined which indicated that there were at least three serological subtypes of IBDV prevalent in this region since the year 2000. The dominant strains in the field belonged to subtype 1 and minor antigenic differences existed among subtype 1 viruses. The commonly used commercial vaccine strains B87 and FW2512 belonged to subtype 1 but with differential antigenicity from most of the field strains. The antigenic diversity among field IBDVs revealed by this study may greatly contribute to the control of this disease.

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