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Novel variant strains of infectious bursal disease virus isolated in China

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ABSTRACT

Infectious bursal disease (IBD) is one of the most important immunosuppressive diseases that seriously threaten poultry farming and food safety worldwide. The variant strain of infectious bursal disease virus (IBDV) has been greatly neglected for more than 30 years. Recently, the subclinical infection of suspected IBD, causing considerable economic losses, occurred in the main chicken-farming regions of China. Through RT-PCR, sequencing, and phylogenic analyses, novel variant IBDVs were first identified in six provinces of eastern China. Immunological detection further confirmed the antigenic variation of the Chinese variant IBDVs. The Chinese IBDV variants were obviously different from the American IBDV variants, with less than a 97.7% (VP1) or 98.7% (VP2) amino acid sequence identity. Animal experiments further confirmed the serious threat of the variant IBDVs to chickens, demonstrating irreversible damage to the central immune organ, obvious immunosuppression, and growth retardation. This study not only identified the pandemic nature of the novel variant IBDVs for the first time but also discovered the distinct molecular epidemiological characteristics of these viruses, which will contribute more to the control of the disease.

1. Introduction

In modern society, avian meat and eggs are becoming increasingly important for the human diet. Consequently, chicken food safety issues have emerged as one of the major public health concerns worldwide, as chickens may be contaminated with pathogens and antibiotics. It has been reported that implementation of antibiotic-free poultry farming is very challenging, particularly in the case of the chicken's immune system is compromised by immunosuppression, predisposing chickens to several opportunistic pathogens (Amini et al., 2015; Gaucher et al., 2015; Kurukulsuriya et al., 2016). Therefore, infectious bursal disease (IBD), as one the most important immunosuppressive diseases seriously threatening poultry farming worldwide (Jackwood, 2017; Muller et al., 2003), has been received immense attention with regard to chicken health and food safety.

IBD is caused by infectious bursal disease virus (IBDV), which is a highly contagious RNA virus that belongs to the *Avibirnavirus* genus of the *birnaviridae* family and has a non-enveloped capsid structure containing a double-stranded RNA genome with two segments, A and B (Brown and Skinner, 1996; Muller et al., 2003). Segment A encodes four viral proteins including the two structural proteins VP2 and VP3, the viral protease VP4, and the nonstructural protein VP5 (Raja et al., 2016). VP2 is the only component of the icosahedral capsid and it is related to virulence, cell tropism, and antigenic variation (Brandt et al., 2001; Jackwood et al., 2008; Qi et al., 2009, 2015, 2016). The amino acid (aa) residues 206-350 of VP2 has been identified as the hypervariable region (HVR) and contains four hydrophilic regions, including aa 210-225 (peak A), 247-254 (minor peak 1), 281-292 (minor peak 2), and 312-324 (peak B) (Boot et al., 2000; Letzel et al., 2007). VP2 is folded into three distinct domains, including base (B), shell (S), and projection (P) domain (Birghan et al., 2000; Garriga et al., 2006; Lee et al., 2006). The tower-like variable P domain contains four loops,

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namely, the P_{BC} (aa 204-236 of VP2), P_{DE} (aa 240-265), P_{FG} (aa 270-293), and P_{HI} (aa 305-337) (Coulibaly et al., 2005). Segment B encodes VP1, as an RNA-dependent RNA polymerase, which plays an important role in viral replication and genetic evolution (Escaffre et al., 2013; Gao et al., 2014; von Einem et al., 2004; Yu et al., 2013). IBDV has two serotypes. Serotype I viruses are pathogenic to chickens, whereas serotype II viruses, isolated from turkeys, are nonpathogenic to chickens. As an RNA virus, IBDV is prone to mutations. Since the identification of the classic strain during the first outbreak of IBD in 1957 (Cosgrove, 1962), an antigenic variant (Jackwood and Saif, 1987) and a very virulent IBDV (vvIBDV) strain (Chettle et al., 1989) have successively emerged and brought new challenges. vvIBDV targets the chicken central immune organ, bursa of Fabricius (BF), and then acutely kills the chicken, while the surviving chickens present severe immunosuppression. Since the 1990s, throughout the world, a high percentage of the strains detected in the field have been vvIBDV, since most samples were taken in the case of acute mortality (de Wit et al., 2018; Jackwood, 2017; Muller et al., 2003; van den Berg et al., 2000). Usually, sampling to detect subclinical infections is quite uncommon, which leads to the underdetection of IBDV strains that cause less or hardly any mortality but might cause serious immunosuppression, such as variant strains (de Wit et al., 2018; Jackwood et al., 2006; Letzel et al., 2007; Sapats and Ignjatovic, 2000). Recently, it was reported that most IBDV strains circulating in North America are variant strains, which cause an enormous economic loss because of a direct or indirect impact (Ojkic et al., 2007; Zachar et al., 2016).

To date, to our knowledge, no major epidemics of variant IBDVs have been reported in Asia. In China, although vvIBDV is being well controlled through reasonable immunization practices and raising management, sporadic outbreaks of IBD are still reported, and immunosuppression-associated losses have gained much attention. However, the prevalence of variant IBDV in China has not been systematically studied. The objectives of this study were to identify the incidence of variant IBDV infection and to evaluate the associated threats to the broiler chicken in the major chicken-farming provinces of China.

2. Materials and methods

2.1. Clinical samples

Since 2015, variant IBDVs were detected in at least 10 provinces in eastern China, which are the main chicken-farming regions in China. The suspected subclinical infections of IBDV have been causing considerable economic losses in several broiler farms in eastern China especially since September 2017. The affected flocks had a dramatically higher morbidity rate, poor feed conversion ratio, and decreased meat production. Bursal atrophy was observed by postmortem examination. In this study, a total of 356 clinical samples of bursae were collected from broilers in 76 immunized farms from 6 provinces (Hebei, Shandong, Shanxi, Anhui, Jiangsu, and Fujian) (Table 1). The bursal tissues were used to prepare a 10% (w/v) homogenate in phosphate-buffered saline (pH 7.2) with added penicillin and streptomycin. The homogenate was frozen and thawed three times followed by centrifugation at 5 000 × g for 5 min at 4 °C, and then the supernatant was harvested for subsequent detection.

2.2. Animals

Specific-pathogen-free (SPF) chickens were purchased from the Experimental Animal Center of the Harbin Veterinary Research Institute (HVRI), the Chinese Academy of Agricultural Sciences (CAAS) and were housed in negative-pressure-filtered air isolators. All the animal experiments were approved by the HVRI of the CAAS and were performed in accordance with the animal ethics guidelines and approved protocols.

2.3. Viral RNA extraction and RT-PCR

The total viral RNA was extracted from the pathologic bursal samples using the Purelink^{TM}RNA Mini kit (Invitrogen, USA). cDNA was synthesized from the viral RNA with M-MLV reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA) and the random primer pd(N)9. The PCR was performed using the forward primer 2U (5'-CCTCAGCTTACCCACATC-3') and the reverse primer 2 L (5'-CCTTCCCCAATTGCATGG-3'), which amplified one 930-bp fragment covering the HVR of the VP2 gene. Moreover, the forward primer B464U (5'-AGGAGAAGCCCAATGCGT-3') and the reverse primer B1718 L (5'-GTCATCAATGGACCTCTC-3') were used to amplify one 1255-bp fragment of the VP1 gene. The PCR was performed at 95 °C for 5 min followed by 35 cycles at 95 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min and 10 s, and a final extension at 72 °C for 10 min.

2.4. Sequencing

The RT-PCR products were purified with the AxyPrep^{TM} DNA Gel Extraction kit (Axygen, USA) and were sequenced by Comate Biosciences Company (Changchun, China). The sequence of a 777-bp fragment (bp 547-1323), corresponding to aa 183-441 covering the HVR (aa 206-350) of VP2, and the sequence of a 1152-bp fragment (bp 511-1662), corresponding to aa 134-517 of VP1, were identified and submitted to GenBank (Table 1).

2.5. Sequence analysis

Except for the samples that contained an insufficient amount for sequencing, 50 IBDV isolates were selected for the sequence analysis of both VP1 and VP2 (Table 1). The sequences of 5 variant, 10 very virulent, 9 attenuated, 1 classic, and 2 serotype II IBDV strains from GenBank were selected as the reference strains. The alignment and phylogenetic analyses based on the nucleotide or amino acid sequences were performed using the Clustal X program (version 2.0) (Larkin et al., 2007) and the MEGA program (version 3.1) (Kumar et al., 2004), and the confidence levels were assessed using 1000 bootstrap replications. The GenBank accession numbers and the nation of the reference strains used in this study are as follows: Variant strains, Variant E (USA) (AF133904, AF133905), Variant A (USA) (M64285), GLS (USA) (AY368653, AY368654), E Del (USA) (DD187400), 9109 (USA) (AY462027, AY459321); vvIBDV, BD399 (Bangladesh) (AF362776, AF362770), 02015.1 (France) (AJ879932, AJ880090), D6948 (Netherlands) (AF240686, AF240687), Gx (China) (AY444873, AY705393), Harbin-1 (China) (AF092171, AF454945), HLJ0504 (China) (GQ451330, GQ451331), HK46 (China) (AF092943, AF092944), OKYM (Japan) (D49706, D49707), UK661 (UK) (NC-004178, NC-004179), YS07 (China) (FJ695138, FJ695139); Attenuated strains, CEF94 (Netherlands) (AF133904, AF133905), CT (France) (AJ310185, AJ310186), CU-1 (Germany) (X16107, AF362775), D78 (USA) (AF499929, AF499930), Gt (China) (DQ403248, DQ403249), HZ2 (China) (AF321054, AF493979), JD1 (China) (AF321055, AY103464), NB (China) (AY319768, AY654284), P2 (Germany) (X84034, X84035); Classic strain, IM (USA) (AY029166, Y029165); Serotype II, 23/82 (Germany) (AF362773, AF362774), OH (Canada) (U30818, U30819).

2.6. Immunofluorescence assay

To determine the monoclonal antibody (MAb) reactivity pattern of the Chinese variant IBDVs, the representative variant IBDV, designated SHG19, was isolated as described previously (Yuwen et al., 2008). Then SHG19 was selected for immunofluorescence assay (IFA) by reacting with a series of MAbs against IBDV VP2. Meanwhile, the reference IBDVs (vvIBDV Gx strain and attenuated Gt strain) were used as control. For IFA, DT40 cells, cultured in a 96-well tissue culture plate, were separately infected with the variant IBDV SHG19, Gx, and Gt strain.

Table 1
IBDV strains isolated in this study.

No	Strain	Origin	Collection date	Host	Age	Genbank accession no.	
						Partial VP1	Partial VP2
1	SHG3	Pingdu in Shandong	201709	Broiler	25d	MH879036	MH879083
2	SHG4	Pingdu in Shandong	201709	Broiler	25d	MH879037	MH879084
3	SHG5	Pingdu in Shandong	201709	Broiler	25d	MH879038	MH879085
4	SHG6	Pingdu in Shandong	201709	Broiler	25d	MH879039	MH879086
5	SHG7	Pingdu in Shandong	201709	Broiler	25d	MH879040	MH879087
6	SHG8	Pingdu in Shandong	201709	Broiler	25d	MH879041	MH879088
7	SHG12	Chuzhou in Anhui	201709	Broiler	36d	MH879042	MH879089
8	SHG13	Chuzhou in Anhui	201709	Broiler	36d	MH879043	MH879090
9	SHG14	Chuzhou in Anhui	201709	Broiler	36d	MH879044	MH879091
10	SHG19	Chuzhou in Anhui	201709	Broiler	36d	MH879045	MH879092
11	SHG20	Chuzhou in Anhui	201709	Broiler	36d	MH879046	MH879093
12	SHG21	Chuzhou in Anhui	201709	Broiler	36d	MH879047	MH879094
13	SHG23	Chuzhou in Anhui	201709	Broiler	36d	MH879048	MH879095
14	SHG25	Chuzhou in Anhui	201710	Broiler	36d	MH879049	MH879096
15	SHG26	Liaocheng in Shandong	201710	Broiler	31d	MH879050	MH879097
16	SHG27	Liaocheng in Shandong	201710	Broiler	31d	MH879051	MH879098
17	SHG28	Liaocheng in Shandong	201710	Broiler	31d	MH879052	MH879099
18	SHG34	Liaocheng in Shandong	201710	Broiler	31d	MH879053	MH879100
19	SHG41	Liaocheng in Shandong	201710	Broiler	31d	MH879054	MH879101
20	SHG43	Qingdao in Shandong	201711	Broiler	25d	MH879055	MH879102
21	SHG44	Qingdao in Shandong	201711	Broiler	25d	MH879056	MH879103
22	SHG49	Qingdao in Shandong	201711	Broiler	24d	MH879057	MH879104
23	SHG53	Qingdao in Shandong	201711	Broiler	25d	MH879058	MH879105
24	SHG78	Yanggu in Shandong	201711	Broiler	32d	MH879059	MH879106
25	SHG83	Laiyang in Shandong	201712	Broiler	26d	MH879060	MH879107
26	SHG84	Laiyang in Shandong	201712	Broiler	26d	MH879061	MH879108
27	SHG115	Jinzhong in Shanxi	201801	Broiler	28d	MH879062	MH879109
28	SHG120	Chuzhou in Anhui	201803	Broiler	40d	MH879063	MH879110
29	SHG121	Chuzhou in Anhui	201803	Broiler	40d	MH879064	MH879111
30	SHG132	Chuzhou in Anhui	201803	Broiler	40d	MH879065	MH879112
31	SHG141	Chuzhou in Anhui	201803	Broiler	40d	MH879066	MH879113
32	SHG144	Bengbu in Anhui	201805	Broiler	36d	MH879067	MH879114
33	SHG165	Rudong in Jiangsu	201806	Broiler	40d	MH925084	MH925087
34	SHG213	Laiyang in Shandong	201806	Broiler	35d	MH879068	MH879116
35	SHG226	Laiyang in Shandong	201806	Broiler	35d	MH879069	MH879117
36	SHG230	Laiyang in Shandong	201806	Broiler	35d	MH879070	MH879118
37	SHG232	Laiyang in Shandong	201806	Broiler	35d	MH879071	MH879119
38	SHG241	Laiyang in Shandong	201806	Broiler	35d	MH879072	MH879120
39	SHG250	Laiyang in Shandong	201806	Broiler	35d	MH879073	MH879121
40	SHG308	Tangshan in Hebei	201807	Broiler	35d	MH879074	MH879122
41	SHG311	Tangshan in Hebei	201807	Broiler	34d	MH879075	MH879123
42	SHG316	Tangshan in Hebei	201807	Broiler	35d	MH879076	MH879124
43	SHG321	Tangshan in Hebei	201807	Broiler	35d	MH879077	MH879125
44	SHG326	Tangshan in Hebei	201807	Broiler	35d	MH879078	MH879126
45	SHG334	Tangshan in Hebei	201807	Broiler	35d	MH879079	MH879127
46	SHG338	Tangshan in Hebei	201807	Broiler	35d	MH879080	MH879128
47	SHG350	Tangshan in Hebei	201807	Broiler	34d	MH879081	MH879129
48	SHG352	Tangshan in Hebei	201807	Broiler	35d	MH879082	MH879130
49	IBD18FJ01	Nanping in Fujian	201809	Broiler	34d	MH925085	MH925088
50	IBD18FJ02	Nanping in Fujian	201809	Broiler	34d	MH925086	MH925089

Cells were treated with PBS as negative control. At 24 h post-infection, the cells were fixed and then incubated with eight MAbs (1-2C-7C, 1-6H-3 A, 2-3B-5D, 2-5C-6 F, 7D4, 8 G, 4-5D-2E, and 3-10H-7 A) for 1 h, respectively, followed by staining with a fluorescein-labeled goat anti-mouse antibody (1:200 solution) (Sigma, USA) for another 1 h. The cells were examined by fluorescence microscopy after being washed five times with PBS. The MAbs were developed, and the reference IBDVs (vvIBDV Gx strain and attenuated Gt strain) were conserved in Division of Avian Infectious Diseases, HVRI, CAAS.

2.7. Pathogenicity experiment

To evaluate the virulence of the variant IBDVs, animal experiment was performed using the representative variant strain SHG19. Sixteen-day-old SPF chickens were randomly divided into three groups. The first group (n = 10) and the second group (n = 15) were infected with 8×10^6 viral RNA copies (200 µl) of SHG19 via the ocular and intranasal routes. The first group was only used to evaluate the mortality rate of SHG19 infection. The third group (n = 25) were received 200 µl of DMEM as a negative control. The chickens were observed daily for clinical symptoms. From 1 to 5 day post-inoculation (d p.i.), three chickens randomly selected each day from the second and third groups were euthanized for necropsy and examination of pathological changes. The bursa and body weights of all the chickens were determined, and the bursa:body weight index (BBIX) on each day was calculated along with the standard deviation [BBIX = (bursa:body weight ratios)/(bursa:body weight ratios in the negative group)]. The mean values and standard deviations of the data obtained from three independent chicken samples were calculated. Bursae with a BBIX less than 0.70 were considered as atrophy (Lucio and Hitchner, 1979). Some part of bursa of each chicken was fixed immediately in 10% neutral buffered formalin and was stained with hematoxylin and eosin for further histopathological examination. Meanwhile, the spleen/body weight ratios were also calculated.

2.8. Horizontal transmission experiment

To simultaneously evaluate the horizontal transmission potential of the variant IBDV in the above animal experiment, 5 additional uninfected chickens with the same background were cohabitated with the 10 infected chickens of the first group in the same isolator. The remaining 10 chickens in the third group were used as controls. As described above, the chickens were observed daily for clinical symptoms. At 25 day post-cohabitation (d p.c.), the body weight, BBIX, spleen/body weight ratio, and pathological changes in the bursa of five chickens selected in each group were examined. In addition, the viral RNA extracted from the bursae of the cohabitation group was assessed by real-time RT-PCR as described previously (Wang et al., 2009). The mean values and standard deviations of five independent chicken samples were calculated.

2.9. Immunosuppression experiment

To determine whether the variant IBDV induced immunosuppression of chickens, the influence of SHG19 infection on vaccination against avian influenza was evaluated. Sixteen-day-old SPF chickens were randomly divided into three groups with each group having 10 chickens. The first group was infected with 8×10^6 viral RNA copies (200 μl) of SHG19 per chicken via the ocular and intranasal routes. At 4 d p.i., the first and second groups were immunized with the recombinant highly pathogenic avian influenza virus (HPAIV) bivalent inactivated vaccine (H5 + H7) (Harbin Weike Biotechnology Development Company, China) (300 μl per chicken) by an intramuscular injection in accordance with the instructions. The H5N1 and H7N9 AIV antigens were included in the bivalent inactivated vaccine. The third group, without infection and vaccination, was used as the control. The chickens were observed daily for clinical symptoms for 14 days. At 0, 7, and 14 day post-vaccination, the serum antibodies against H5 and H7 were detected by means of hemagglutination assays (HAs).

2.10. Statistical analyses

A one-way ANOVA was employed to evaluate the significance of the differences among the different groups. Differences with $P < 0.05$ were considered significant.

3. Results

3.1. IBDV detection

From 76 broiler flocks in six provinces of eastern China (Table 1), a total of 356 bursal samples with suspected IBD were assessed by RT-PCR, and 52.5% (187/356) of the samples were positive for IBDV, from which 50 representative samples were selected to sequence both partial fragments of VP1 and VP2. The representative fragment sequences of both VP1 and VP2 of all 50 variant strains were submitted to GenBank, and the accession numbers are summarized in Table 1.

3.2. Chinese isolates evolutionarily belong to the variant IBDV

A phylogenetic tree, based on the amino acid sequences of the representative fragment of VP2, showed that the IBDV strains were distinctly divided into five major branches, including classic strains, variant strains, very virulent strains, attenuated strains, and serotype II strains. All 50 Chinese strains isolated in the study formed one branch with the reference strains of the variant IBDVs, including the Variant E, GLS, Variant A, E/Del, and 9109 strains from America (Fig. 1A). Based on the partial amino acid sequence of VP2, all 50 Chinese strains had 95.8-97.7% identity with the variant IBDV reference strains (except for the GLS strain), which is comparatively higher than that with the vvIBDV (93.5-95%), the classic strain (94.2-95.4%), and the attenuated strain (92.7-94.2%). The HVRs of VP2 were further compared, which showed that all 50 Chinese strains had the same characteristic amino acids as the reference variant strain Variant E, including 213 N, 222 T, 242 V, 249 K, 253Q, 279 N, 284 A, 286I, 294 L, 318D, 323E, and 330S, and among these, 222 T, 249 K, 286I, and 318D are the typical residues of the variant IBDVs (Jackwood, 2012; Jackwood et al., 2006).

Regarding the representative fragment of VP1, the phylogenetic tree based on the amino acid sequences was divided into three major branches, including the serotype II strain branch, the vvIBDV branch, and the third branch encompassing the variant strains, classic strains, and attenuated strains (Fig. 1B). All 50 Chinese strains had a comparatively higher identity, at 97.1-98.4%, with the reference strains of variant IBDVs than that with vvIBDV (94.8-97.4%). All 50 Chinese strains had the same characteristic amino acids as the reference variant strain Variant E, including 145 N, 146E, 242D, 287 T, 390 L, and 511R. Taken together, all 50 Chinese strains in this study evolutionarily belonged to the variant IBDVs.

3.3. The Chinese variant IBDVs have distinct genetic characters

Although the Chinese variant IBDV strains were in the same branch as the American reference strains, these Chinese variants showed obvious differences compared to the American variants of IBDV. As showed in Fig. 1, in the branch of the variant IBDVs, the Chinese variants and the American variants formed two distinct sub-branches. The Chinese variants showed only 96.9-97.7% amino acid sequence identity to the variant reference strain Variant E, 96.5-97.3% to E/Del, 95.8-96.9% to Variant A, 95.8-96.9% to 9109, and 94.2-95.4% to GLS. In the sub-branch of the Chinese variants, two groups were observed. The variant strains isolated from the northern provinces (Shandong and Hebei) formed group I, while group II was a combination of isolates from the southern provinces (Anhui, Jiangsu, and Fujian) and northern provinces (Fig. 1A). Notably, in the HVR, three conserved amino acid residues including 217 K, 252I, and 299S only existed in Chinese variant IBDVs, which serve as distinct features distinguished from the American variants.

In the phylogenetic tree constructed by the partial amino acid sequence of VP1, although the Chinese variant IBDVs were in one branch with the American variant strains, classic strains, and attenuated strains, the Chinese variant IBDVs formed one distinct sub-branch, which showed less than 98.7% identity to the other strains. Moreover, compared to the American variant IBDVs, two distinct conserved amino acid residues (147D and 508 K) were observed in the representative fragment of VP1. Thus, these Chinese strains were evolutionary variants of IBDV with distinct genetic characters.

3.4. A Chinese variant IBDV has a distinct MAb reactivity pattern

The representative strain SHG19 of the Chinese variant IBDVs reacted with only one MAb, namely, 7D4, while the non-variant IBDVs, including vvIBDV and the attenuated strain, were recognized by all 8 MAbs (Fig. 2), which further confirmed the antigenic variation of the Chinese IBDV variants in this study.

3.5. Variant IBDV is pathogenic to chickens

Compared to the control, no gross clinical symptoms or mortality was observed in the chickens of the first group infected with the IBDV variant SHG19. However, the body weight was negatively influenced ($P < 0.01$) at 25 d p.i. (Fig. 3A). To further assess via autopsy the lesions induced by SHG19, the infected chickens in the second group were randomly selected for necropsy, and inflammatory exudation, hemorrhage, or yellow staining of the bursa were observed from 3-5 d p.i. The BBIX in the second group was below 0.7 at 3 d p.i. and then continued to decrease to 0.24 until 5 d p.i. (Fig. 3B), which indicated

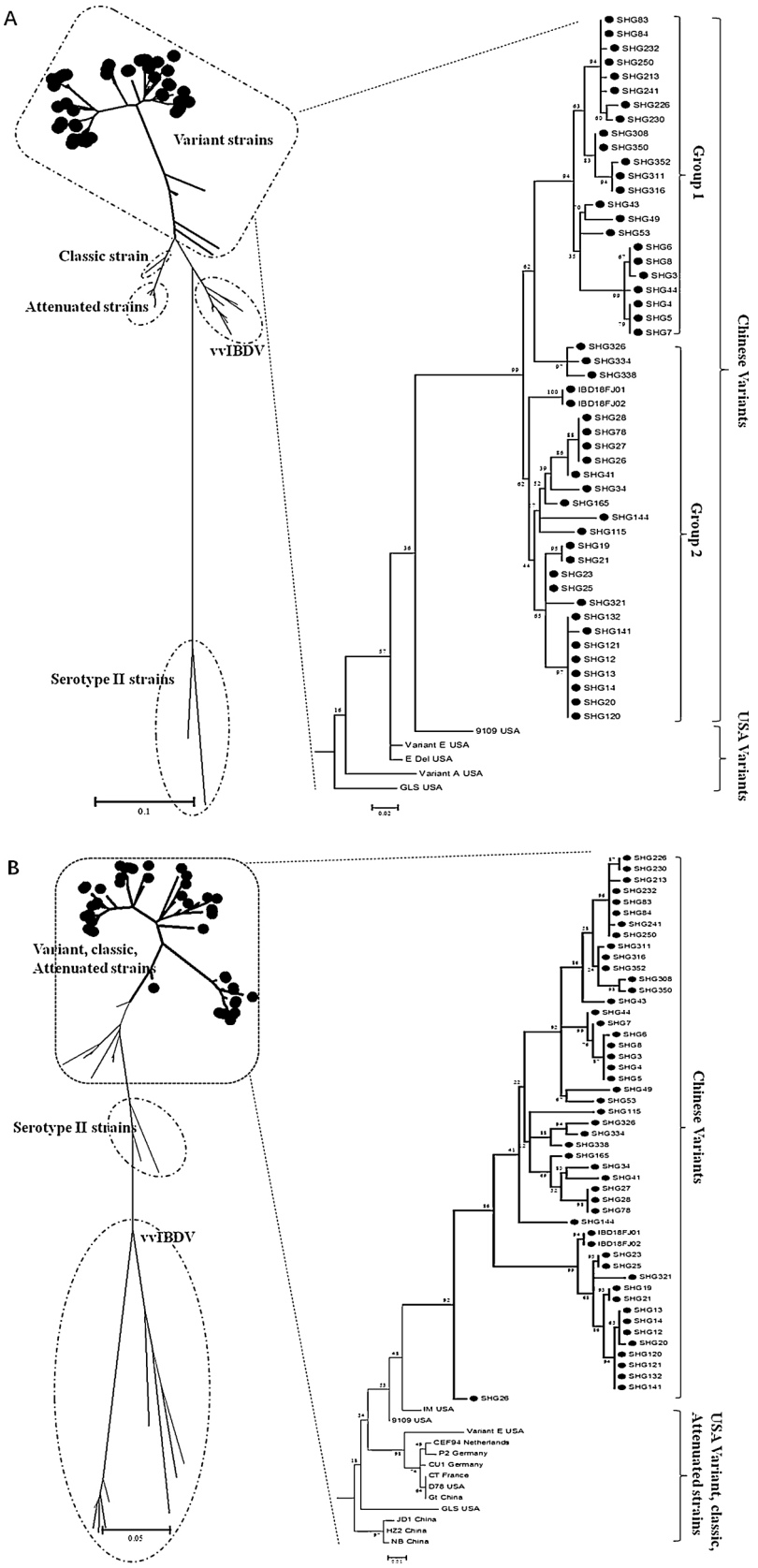

Fig. 1. Phylogenetic tree analysis of the amino acid sequence of the representative partial fragments of VP2 (aa 183-441) (A) and VP1 (aa 134-517) (B). The trees were generated by the neighbor-joining method using MEGA6. The variant strains detected in this study are highlighted with a solid circle.

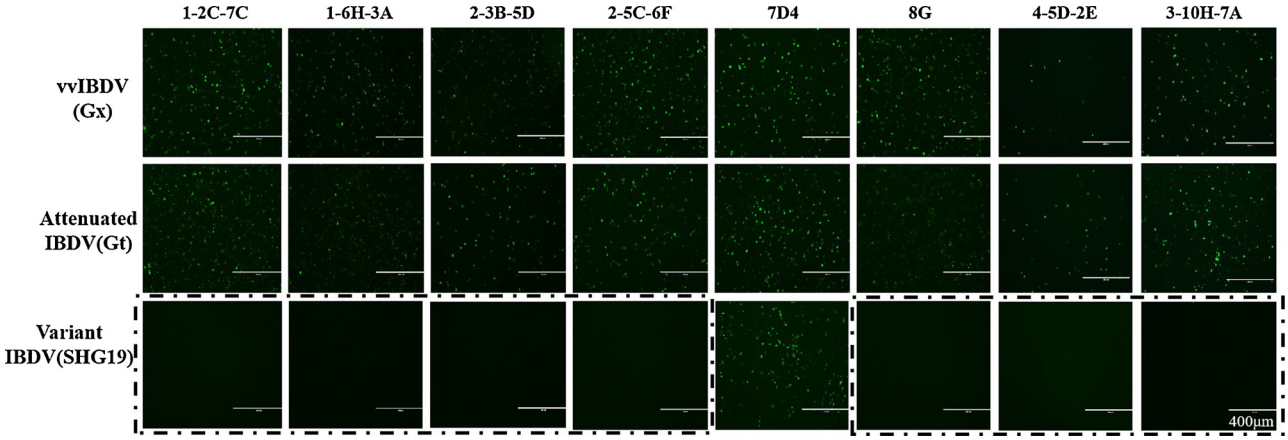

Fig. 2. MAb reactivity pattern of the Chinese variant IBDV. The immunofluorescence assay on the DT40 cells involving a series of anti-IBDV VP2 MAbs was performed to detect the MAb reactivity pattern of the variant IBDV (SHG19) compared to the vvIBDV (Gx) and attenuated strain (Gt). The negative reactivity groups are highlighted with a dotted box. The scale bars represent 400 μm.

that the process of bursal atrophy was induced by SHG19. In addition, the spleen/body weight ratio of the SHG19-infected chickens in the second group was higher than that of the control, and the difference was significant, especially after 4 d p.i. (Fig. 3C). Furthermore, the histopathological lesions of the infected bursae, at different day p.i., are presented (Fig. 3D). From 1 d p.i., the lymphocytes obviously decreased, and macrophage infiltration was observed in the follicle. From 3 d p.i., the proliferation of fibrous tissue was observed around the follicle. At 5 d p.i., severe atrophy of the follicle was observed. No pathological lesions were found in the control group (Fig. 3D). This data confirmed that the variant IBDV SHG19 was highly pathogenic to chickens.

3.6. Variant IBDV is prone to horizontal transmission

The cohabitated uninfected chickens with the SHG19-infected group were sacrificed at 25 day post-cohabitation (d p.c.) for assessment of horizontal transmission of IBDV. Firstly, IBDV specific antibodies were

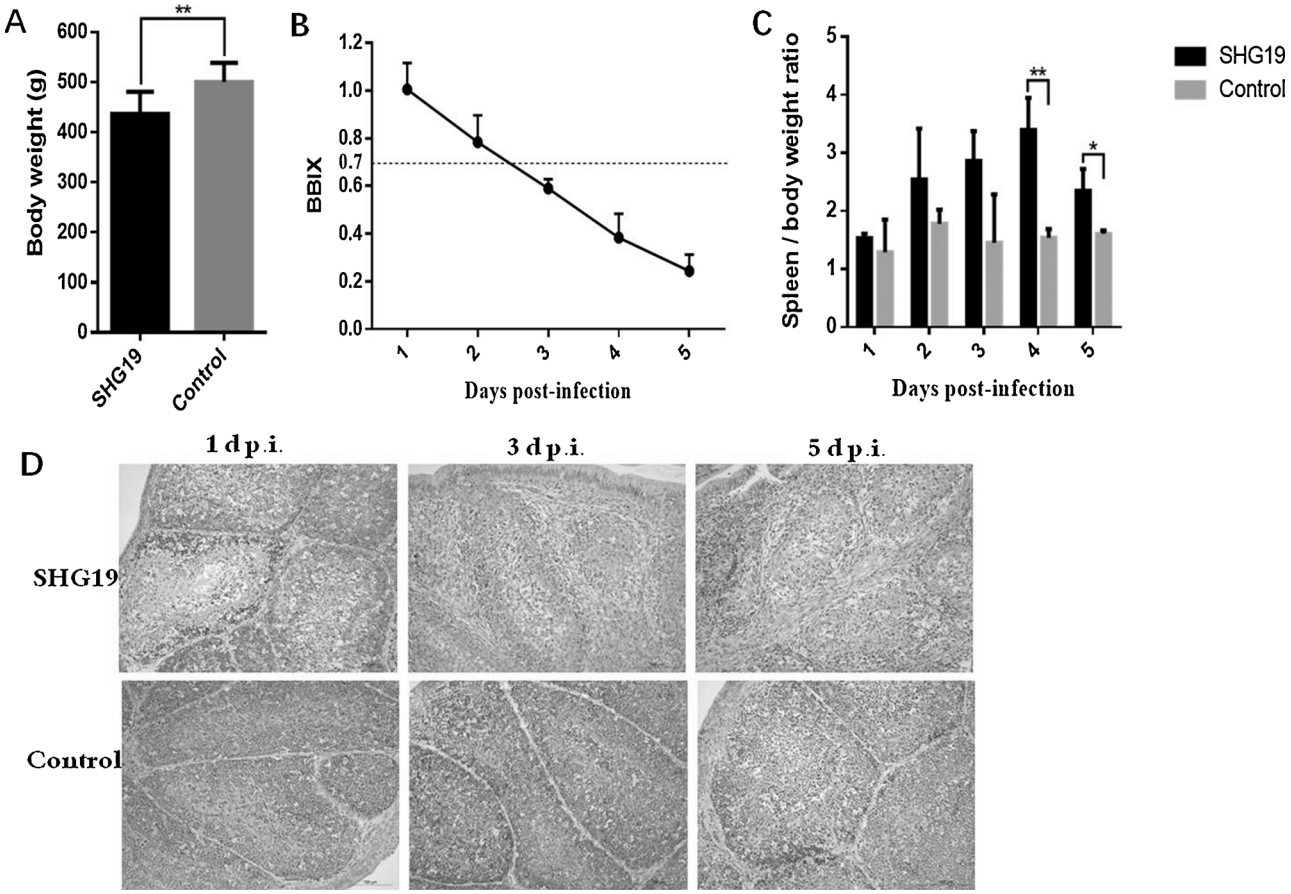

Fig. 3. Evaluation of the virulence of the IBDV variant strain SHG19 using SPF Chickens. A. The body weight at 25 d post-infection (p.i.). B. The kinetics curve of the BBIX. C. The spleen/body weight ratio. D. The histopathological appearance of the bursal sections (hematoxylin and eosin staining). The average titers and standard deviations (error bars) from ten (A) or three (B, C) independent samples are shown.

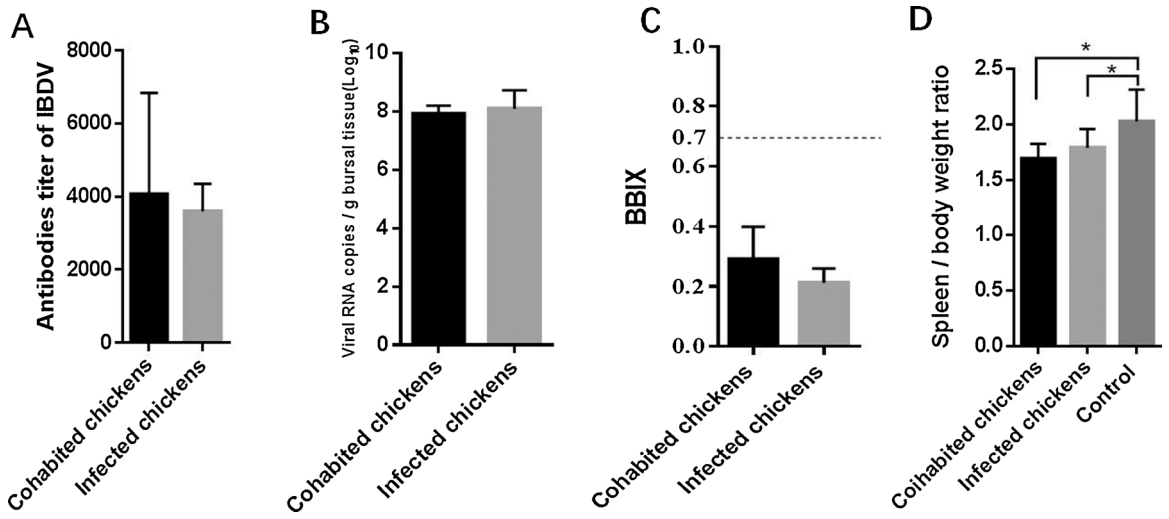

Fig. 4. Evaluation of the horizontal transmission ability of the IBDV variant strain SHG19. A. IBDV specific antibodies at 25 day post-cohabitation (p.c.). B. Viral load of IBDV in the bursa at 25 d p.c. C. BBIX at 25 d p.c. D. The spleen/body weight ratio at 25 d p.c. The average titers and standard deviations (error bars) from five independent samples are shown.

detected by an ELISA, which suggested the occurrence of horizontal transmission of the variant IBDV SHG19 (Fig. 4A). Secondly, IBDV replication was further confirmed in the bursae of the cohabitated chickens collected at 25 d p.c., in which the average titer reached 7.9×10^7 viral RNA copies/g tissue (Fig. 4B). Meanwhile, severe atrophy of the bursa, with an average BBIX of 0.29, was also observed in the cohabitated chickens (Fig. 4C). Moreover, the spleen/body weight ratio of the cohabitated chickens was significantly lower than that of the control (Fig. 4D). These data indicated that the IBDV variant SHG19 was prone to horizontal transmission and then severely damaged the bursa and spleen.

3.7. Variant IBDV induces immunosuppression on AIV vaccination

At 4 d p.i., vaccination with a recombinant AIV bivalent inactivated vaccine (H5 + H7) was used to evaluate the immunosuppression by the variant IBDV SHG19. Seroconversion was detected at 14 day post-vaccination. Compared with that observed in the vaccinated chickens without SHG19 infection in the first group, the HI titer of the AIV antibodies against both H5 and H7 was obviously suppressed by SHG19 infection in the second group (Fig. 5).

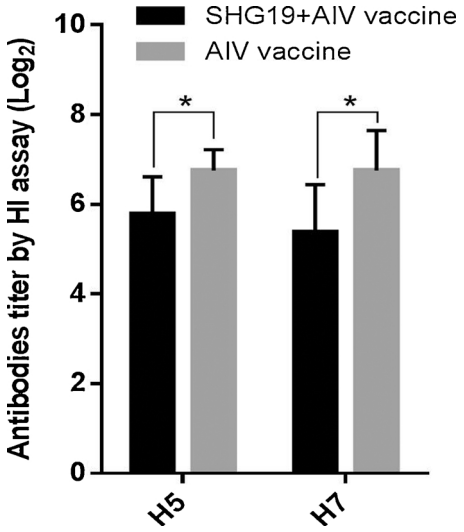

Fig. 5. Antibody titer to the AIV vaccine determined by a homologous HI assay. The average titers and standard deviations (error bars) from ten independent samples are shown.

4. Discussion

Following the first emergence of classic IBDV in America in 1957 (Cosgrove, 1962), one antigenic variant (referred to as variant IBDV) was reported in the late 1980s in America, which escaped from the immune protection against classic IBDV (Geerligs et al., 2015; Jackwood and Saif, 1987). This variant IBDV has been reported as an economically significant disease worldwide because it induced severe bursal damage and resulted in profound immunosuppression and subclinical infections, which are often the underlying cause of respiratory and enteric diseases in chickens, as well as vaccination failures (Icard et al., 2008; Jackwood and Sommer-Wagner, 2005; Kurukulsuriya et al., 2016; Perozo et al., 2009). However, over the past 30 years, variant IBDV was inadvertently neglected because of the ensuing cases of vvIBDV (de Wit et al., 2018; Jackwood et al., 2006; Letzel et al., 2007). Due to the acute high mortality with huge economic losses of this very virulent strain, more attention has been paid to the study of and protection from vvIBDV. As a result, there are few research reports about variant IBDVs worldwide, except for in North America (Jackwood, 2012; Kurukulsuriya et al., 2016; Letzel et al., 2007; Zachar et al., 2016), Europe (Jackwood et al., 2006), and Australia (Sapats and Ignjatovic, 2000).

Since 2017, subclinical infections of suspected IBD have occurred in several broiler farms in eastern China and caused considerable economic losses. It is worth mentioning that many variant IBDVs were observed in these samples, which covered the main chicken-farming regions. Representative fragments of both VP1 and VP2 of 50 variant strains were cloned, sequenced, and submitted to GenBank, which greatly enriched the variant IBDV information in GenBank. Due to a lack of submissions, only a very few sequences cover both segments of the variant IBDVs, such as Variant E, 9109, and GLS, that existed in GenBank. To our knowledge, this is the first report of a large-scale epidemic caused by variant IBDV in Asia since the 1990s.

It is well known that VP2 is the antigenic determinant and is most commonly used for the molecular characterization of IBDV (Brandt et al., 2001; Jackwood, 2017; Jackwood et al., 2008; Yuwen et al., 2008). Recently, VP2 was used for the identification and analysis of variant IBDV (de Wit et al., 2018; Gelb et al., 2012; Jackwood, 2012; Jackwood et al., 2018; Jackwood and Sommer-Wagner, 2005; Lana et al., 1992; Zachar et al., 2016). In this study, a sequence analysis

based on the representative fragment of VP2 showed that all 50 Chinese strains formed one branch with the American variant IBDV, and these strains had the same typical residues as the variant IBDV, including 222T, 249K, 286I, and 318D (Jackwood, 2012; Jackwood et al., 2006). Although VP2 is the antigenic determinant (Brandt et al., 2001; Jackwood et al., 2008), a representative fragment of VP1 was also analyzed, which was very essential to fully understand the molecular characteristics of IBDV with two genome segments (He et al., 2014; Islam et al., 2012). In the phylogenetic tree based on the partial VP1 sequence, all 50 Chinese strains were also in the same branch as the American IBDV variants. These data suggested that all 50 strains in this study evolutionarily belonged to the variant IBDV. To verify this association, an indirect immunofluorescence assay was performed by using a representative variant strain SHG19 to react with a series of anti-VP2 MAbs. The distinct pattern of viral MAb reactivity further confirmed the antigenic variation of the Chinese variant IBDVs isolated in this study.

Among the characteristic amino acids observed in the variant IBDVs, including the Chinese isolates, most of the residues were located in the hydrophilic peaks of the HVR in VP2, which have been found to influence the antigen and the MAb reactivity pattern of IBDV (Jackwood and Sommer-Wagner, 2011; Letzel et al., 2007; Vakharia et al., 1994). Residue 222, in the hydrophilic peak A of VP2 is located in the P_{FG} loop and is closely related to viral antigenicity because A222T or P222S changes the reactivity of the corresponding epitope with MAb 67 (Letzel et al., 2007). One vaccine strain, Del-E, even broke through maternal immunity because of the natural mutation T222 A (Jackwood and Sommer-Wagner, 2011). Residues 318, 321, and 323 (in P_{HI}) in the hydrophilic peak B and residue 286 (in P_{FG}) in the minor peak 2 of VP2 are involved in the reactivity of both MAb 57 and MAb 67 (Letzel et al., 2007; Vakharia et al., 1994). In the binding of MAb B69, residue 249 (in P_{DE}) in minor peak 1 of VP2 may be the critical residue (Letzel et al., 2007; Vakharia et al., 1994). Residues 311, 320, and 326 (in P_{HI}) in peak B are involved in the recognition of MAb 179. The mutation of residue 254 (in P_{DE}) in minor peak 1 of VP2 also contributes to the antigenic drift of IBDV (Jackwood and Sommer-Wagner, 2011). Compared with the non-variant strains, the residue exchanges A222T, Q249K, G254N, T286I, G318D, and D323E are observed in the Chinese variant IBDV strains, including the SHG19 strain. The exact relationship between the distinct genetic characters and the special MAb reactivity patterns of the Chinese variant IBDVs will be explored further.

In addition, although the Chinese variant IBDVs clustered into the branch of the variant IBDV, they were obviously different from the American variant IBDVs with distinct molecular characteristics (Fig. 1). All the Chinese variant IBDVs formed a distinct sub-cluster in the phylogenetic trees of the partial VP1 and VP2 sequences, which showed less than 97.7% (VP1) and 98.7% (VP2) identity to the American variant IBDVs, respectively. Compared with the American variant IBDVs, distinct amino acid residues in VP1 (147D and 508K) and VP2 (221K, 252I, and 299S) were observed in the Chinese variant IBDVs. The amino acid triplets at positions 145/146/147 (TDN, TEG, or NEG) of VP1 are important virulence sites that influence the RNA-dependent RNA polymerase (Gao et al., 2014; Pan et al., 2007). Interestingly, VP1 of the Chinese variant IBDVs had a fourth kind of triplet (NED). Residues 221 and 252 in the HVR of VP2 were located in the P_{BC} and P_{DE} loops of the tip of the viral capsid, which might be involved in the virulence or variation of the Chinese variant IBDVs. Thus, the Chinese variant IBDV strains had distinct evolutionary characteristics.

To evaluate the pathogenicity of the Chinese variant IBDVs, the SHG19 strain was selected to infect 16-day-old chickens in our study. SHG19 did not cause obvious gross clinical symptoms. For subclinical disease, the degree of damage to the bursa has been extensively used as good index of virulence (Jackwood et al., 2008; Qi et al., 2009, 2013). Furthermore, the histopathological changes of the bursa were detected in detail. Interestingly, even at 1 d p.i., an obvious reduction in lymphocytes and the infiltration of macrophages was observed. Usually, obvious pathological lesions in the bursa are only first observed at 2 d p.i. even in vvIBDV-infected chickens (Qi et al., 2009, 2013). The underlying mechanism needs to be further explored. Furthermore, the SHG19-induced lesions in the bursa were irreversible and persisted at least until 25 d p.i. Moreover, SHG19 caused swelling of the spleen, another important immune organ, during early infection (4 - 5 d p.i.) followed by spleen atrophy (at least at 25 d p.i.). Furthermore, our data confirmed the horizontal transmission of SHG19, and the cohabitated chickens also appeared to have severe lesions of the immune organs. Variant IBDV easily spreads, increasing its harm. These data showed that the Chinese variant IBDV induced severe subclinical infection, which was consistent with the epidemic situation in the field.

It was logically speculated that the destruction of the central immune organ by variant IBDV might induce immunosuppression, which is a problem for the poultry industry worldwide (Hoerr, 2010). Our results demonstrated that SHG19 simultaneously suppressed immune responses against both the H5 and H7 HPAIV antigens, which are the immune agents for the two most important viruses threatening poultry farming. SHG19 infection reduced the HI Ab titer against both the H5 and H7 HPAIV antigens in the vaccine. To our knowledge, there are no reports of the evaluation of the efficacy of vaccination with two types of HPAIV antigens when birds are exposed to IBDV. An early infection with variant IBDV induces more severe immunosuppression. In one recent report, 1-day-old chickens exposed to variant IBDV were not well protected by an inactivated H7 AIV vaccine (Spackman et al., 2018). It has been reported that the poor efficacy of AIV vaccines is constantly documented in the field despite good experimental results in the laboratory (Spackman et al., 2018). Thus, variant IBDV-associated immunosuppression needs more attention.

In summary, for the first time, the pandemic novel variant IBDVs in the main chicken-farming regions of eastern China were determined, which revealed one reason for the considerable economic losses because of subclinical infections. The virulence and immunosuppression caused by variant IBDV greatly evokes a new focus on the disease, which is very important for healthy breeding. The identification of the distinct molecular epidemiological characteristics of the Chinese variant IBDVs will contribute to the control of disease.

Conflict of interest statement

The authors declare that they have no competing interests.

Acknowledgments

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