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Residues of 862, 921 of VP3 are associated with virulence in infectious bursal disease virus strain Harbin-1

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ABSTRACT

Reverse genetics was used to study the effect of particular amino acids of infectious bursal disease virus (IBDV) on virulence. Using site-directed mutagenesis, altering of two amino acids in VP2 (Q253H, A284T) and VP3 (H783Q, V862M, I921V) in the segment A of a Chinese very virulent IBDV field strain Harbin-1, 4 virus mutants including $H_{253/284}$, $H_{783/862}$, $H_{862/921}$, $H_{921/783}$ were rescued. To evaluate the characteristics of the recovered viruses in vivo, we inoculated 4-week-old chickens with virus mutants and rescued Harbin-1 (rHarbin-1), analyzed their bursae for pathological lesions 4 days postinfection. rHarbin-1 and $H_{783/862}$, $H_{253/284}$ caused severe bursal lesion, milder lesion for $H_{862/921}$, mildest for $H_{921/783}$. However, $H_{253/284}$ caused the lowest mortality. The results showed that residue at position Q253, A284 of VP2 and V862, I921 of VP3 gene are involved with virulence, but there is difference between VP2 and VP3's role in virulence. The ability of 862 and 921 to control virulence in VP3 is stronger than 253 and 284.

Keywords: IBDV; VP3; Mutagenesis; Reverse Genetics; Virulence

1. INTRODUCTION

Infectious bursal disease (IBD) is a highly contagious disease among young chickens and characterized by the destruction of the bursa of Fabricius. IBD was first described by Cosgrove [1], but in China the first case was reported in 1979 [2]. Nowadays IBD has spread worldwide and continues to threat the poultry industry. Infectious bursal disease virus (IBDV) is the causative agent of the disease, belonging to *Avibirnavirus* genus of the *Birnaviridae* family [3]. Europe had experienced the emergence of very virulent infectious bursal disease virus (vvIBDV) which can cause up to 70% flock mortality [4,5]. Meanwhile, vvIBDV infections also have been observed in Asia and in South America [6].

The genome of IBDV consists of two segments of double-stranded RNA (dsRNA), approximately 3.4 kb (segment A) and 2.7 kb (segment B) in length [7]. Segment A contains two partially overlapping open reading frames (ORFs). The larger ORF encodes a polyprotein (1,012 amino acids, 110 kDa) that is autocatalytically cleaved to yield the viral proteins pVP2 (VPX) (48 kDa), VP4 (29 kDa) and VP3 (33 kDa). During virus maturation, pVP2 is processed into matured VP2 (41 to 38 kDa), probably resulting from site-specific cleavage of pVP2 by a host cell-encoded protease [8]. The smaller ORF encodes the nonstructural protein VP5 (145 to 149 amino acids, 17 kDa). Segment B encodes VP1 (970 kDa) having putative RNA-dependent RNA polymerase activity [9,10]. This protein is covalently linked to the 5' ends of the genomic RNA segments or present at a free form [11,12]. VP2 and VP3 are the major structural protein of the virion. The VP2 is the major host-protective antigen of IBDV and contains the determinants responsible for causing antigenic variation [13-15]. Position 279 and 284 amino acids in the VP2 variable region possibly contribute to virulence of IBDV [16]. Residues 253 and 284 of the VP2 protein of the variant virus are necessary for tissue culture infectivity [17]. The virulence and pathogenic-phenotype markers of IBDV reside in VP2 and residues at position 253 (Gln), 279 (Asp) and 284 (Ala) of VP2 are involved in the virulence and

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pathogenic phenotype of virulent IBDV [18-20]. However, recent study demonstrated VP2 is not the sole determinant of the very virulent phenotype [21]. C-terminal part of VP3 may play a decisive role in controlling the virulence [22]. VP3 could play an important role in receptor-mediated virus-cell attachment, which implied that VP3 has relation with virulence [23].

In order to verify if VP3 have molecular determinant of virulence for Chinese vvIBDV strain Harbin-1, amino acids in VP3 among Harbin-1, D78 (vaccine strain), TY89 (IBDV serotype II) were aligned, the different amino acids among them were listed in **Table 1**. TY89 could not infect B lymphocytes, having no virulence to B lymphocytes, and D78 has mild virulence to B lymphocytes. Based on the result of alignment the amino acids in VP3 that maybe involved in virulence could be found. Position 783 and 862 in Harbin-1 have different amino acids from D78, however, position 921 is different from TY89. To prove their role in virulence, position 783, 862 and 921 in VP3 were mutated subsequently to obtain the combination of two points mutation. As a control, position 253 and 284 in VP2 hypervarible region was mutated at the same time. By use of cRNA-based reverse-genetics system for IBDV [20], four virus mutants were recovered. Furthermore, the characteristics of recovered virus in vitro and in vivo were described and the amino acids responsible for virulence. In this paper we report the discovery that residues of 783, 862, 921 of VP3 are associated with virulence of IBDV.

2. MATERIALS AND METHODS

2.1. Virus and Cells

The very virulent strain Harbin-1 was kindly given by Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences. Harbin-1 causes 100% morbidity and mortality of specific-pathogen-free (SPF) chickens, the mean infection lethal dose (ILD_{50}) for SPF embryo is 10^{-4}/0.2 ml. Primary bursal cells were derived from 18-day-old embryonated SPF eggs (Merial, Beijing, China) and were grown in Dulbecco's minimal essential medium (DMEM, Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS) and maintained with DMEM with 5% FBS [22]. Transfection experiments were performed on primary bursal cells. All virus mutants including $H_{253/284}$, $H_{783/862}$, $H_{862/921}$, $H_{921/783}$ and rHarbin-1, Harbin-1 were used as the viruses for challenge at a dose of 1200 pfu per animal via eye and nose drop.

2.2. Construction of Full-Length CDNA Clones

Several clones for segment A and segment B of Harbin-1 were constructed, pGEM-T-HA (coding sequence of segment A clone), pGEM-T-H5′-A (5′ non-coding sequence of segment A clone), and pGEM-T-H3′A (3′ non-coding sequence of segment A clone), pGEM-T-HB (coding sequence of segment B clone), pGEM-T-H5′B (5′ non-coding sequence of segment B clone), pG EM-T-H3′B (3′ non-coding sequence of segment B clone). All recombinant plasmids were based on pGEM-T (Promega, Madison, WI, USA). There is partly overlapped area between CR (coding region) clone and NCR (non-coding region) clone for segment A and B. But the overlapped area lack appropriate restriction site, thus fusion PCR was used to ligate the NCR and CR to obtain the full length cDNA clone for segment A and B. Oligonucleotides HACR1, HACR2, HANCR1, HAN CR2, HBCR1, HBCR2, HBNCR1, HBNCR2 (**Table 2**) were adopted for segment A and B. For transcription in vitro, EcoRI site and T7 promoter was introduced into 5′ end of oligonucleotides; XbaI site at 3′ end in segment A and XhoI site at 3′ end in segment B. The fusion PCR product of segment A and B was ligated into the T-vector (Takara Bio, Dalian, China) to obtain full-length cDNA clone named as pRHA and pRHB respectively. The sequence of final products was determined by Takara Bio Ltd.

2.3. Site-Directed Mutagenesis

Mutations were introduced into the cDNA of segment A of Harbin-1 according to the manufacture's instruction of QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) with minor modification. Amino acid residues 253, 284, 783, 862, 921 were located in large open reading frame of segment A and their

Table 1. Different AA in VP3 for Harbin-1 and vaccine strain, serotype II strain.

AA site	767	773	783	787	815	862	899	905	921	947	981	990	992	1005	AA site
strain															strain
Harbin-1	S	E	H	S	R	V	D	L	I	K	P	V	T	A	Harbin-1
D78	S	E	Q	S	R	M	D	L	I	K	L	A	T	A	D78
TY89	D	D	R	Q	K	M	E	P	V	R	P	A	S	T	TY89

Table 2. Oligonucleotides used for amplification of Harbin-1 sequence*.

Oligonucleotides	Qrientation	Position	Name
CTCCTCCTTCTACAACGCTATCAT	sense	71-94	HACR1
GAATCTAGAGGGGACCCGCGAACG XbaI	antisense	3246-3260	HACR2
GGAATTCTAATACGACTCACTATAGGGGGGATACGATCGGTCTG	sense	1-20	HANCR1
EcoRI			
GATCTTGCAGGTTCGTCATCGC	antisense	128-149	HANCR2
TGGCTACTAGGGGCGATGC	sense	42-6	HBCR
CTTCTTGAGTGGTTCCCATC	antisense	2756-2775	HBCR2
GGAATTCTAATACGACTCACTATAGGATACGATGGGTCTGACCCT	sense	1-21	HBNCR1
EcoRI			
GTCACTCATGGTGGCAGAATCAT	antisense	98-120	HBNCR2
AAACAAGCGTCCAtGGCCTTATACTGGGTGCTA	sense	876-908	253mut1
TAGCACCCAGTATAAGGCCATgGACGCTTGTTT	antisense	876-908	253mut2
GACAATGGGCTAACGACcGGCACTGACA	sense	965-992	284mut1
TGTCAGTGCCgGTCGTTAGCCCATTGTC	antisense	965-992	284mut2
GACCCACTGTTCCAaTCTGCGCTCAG	sense	2465-2490	783mut1
CTGAGCGCAGAtTGGAACAGTGGGTC	antisense	2465-2490	783mut2
CTCAAAGAAGaTGGAGACTATGGG	sense	2704-2727	862mut1
CCCATAGTCTCCAtCTTCTTTGAG	antisense	2704-2727	862mut2
CATCAGAAGAACAAgTCCTAAGGGCAG	sense	2877-2903	921mut1
CTGCCCTTAGGAcTTGTTCTTCTGATG	antisense	2625-2652	921mut2
TAACCGTCCTCAGCTTACCC	sense	625-644	outside1
TCAGGATTTGGGATCAGCTC	antisense	1246-1265	outside2
CCAACCAGCGAGATAACC	sense	1019-1036	inside 1
GGCGACCGTAACGACAG	antisense	1212-1228	inside 2
TTCTCAGCTAATATCGATGC	sense	842-861	53,84 inupper
GATGTGATTGGCTGGGTT	antisense	1057-1074	53,84 inlower
GTCCAACTGGGCGACGTT	sense	2296-2313	vp3 outupper
CTGGGATTGCGATGCTTCA	antisense	3069-3087	vp3 outlower
CTTCCACCCAATGCAGGAC	sense	2378-2396	783 inupper
CTTTGGCGACTTCGTCTATGA	antisense	2976-2996	62,21 inlower

*Sequence and location of the oligonucleotide used in the study. Underlined nucleotides are virus-specific. Altered nucleotides for mutagenesis are in lowercase, the altered coding nucleotide triplets are highlighted in boldface. Used restriction sites are highlighted in boldface and appropriate restriction enzymes are named. The positions where the primers bind (nucleotide number) are in accordance with the sequence of strain P2 (Mundt *et al.*, 1995).

base sites were in position 893(A→T), 984(G→A), 2483(T→A), 2718(G→A), 2895(A→G) respectively in segment of Harbin-1. First, single site-directed mutagenesis was introduced into the segment A of Harbin-1 with oligonucleotides 253 mut, 783 mut, 862 mut, 921 mut (**Table 2**); the mutants were sequenced to verify the

resultant mutation; after then the second site-directed mutagenesis was introduced into the first mutation product with oligonucleotides 284 mut, 862 mut, 921 mut, 783 mut (**Table 2**) to obtain two point mutagenesis clone named p253/284 m, p783/862 m, p862/921 m, p921/783 m respectively. The second mutation products were sequenced by the company (Takara). The obtained muta-genized plasmids with the alteration of two amino acids, Q253H-A284T, H783Q-V862M, V862M-I921V, H783 Q-I921V were used for subsequent transcription *in vitro* and transfection experiments.

2.4. Transcription and Transfection of Synthetic RNAs

The experiment was performed by the protocol described by Mundt with minor alterations [24]. For transcription *in vitro*, non-mutation and mutated plasmids of segment A and intact segment B were linearized by cleavage with XbaI and XholI respectively. After restrictive digestion, the products were adjusted to 0.5% SDS and incubated with proteinase K (0.5 mg/ml) for 1 hr at 37℃. The linearized DNA templates were recovered by ethanol precipitation, and 1 μg linearized DNA was used for transcription. Segment A and segment B was transcribed respectively. Transcription reaction mixture (30 μl) containing 40 mM Tris-HCl (pH 7.9), 10 mM NaCl, 6 mM $MgCl_2$, 2 mM spermidine, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM UTP, 0.1 mM GTP, 0.25 mM cap analog [m7G(5′)ppp(5′)G] (Promega), 20 units RNasin, 130 units T7 RNA polymerase (Promega), and incubated at 37℃ for 1 hr. As controls, the transcription products were treated with either DNase or RNase (Promega).

After primary bursal cells were grown to 80% confluency in 35-mm dishes, the cells were washed with DMEM (free serum) and incubated at 37℃ for 10 minutes in a CO_2 incubator. The process was repeated again. Simultaneously, 60 μl DMEM (free serum) was incubated with 6 μl of Lipofectin reagent (Invitrogen, Carlsbad, CA, USA) for 60 min in a polystyrene tube at room temperature to form Lipofectin-DMEM mixture. Synthetic RNA transcripts of both segments resuspended in 30 μl of DEPC treated water were mixed and added to the DMEM-Lipofectin mixture, mixed gently and incubated on ice for 5 min. After removing the DMEM from the monolayers in the 35-mm dishes and replacing it with fresh 800 μl of DMEM, the nucleic acid-containing mixture was added drop-wise to the cells and swirled gently. After 2 hours of incubation at 37℃, the mixture was replaced with DMEM containing 5% FCS (without rinsing the cells), and further incubated at 37℃ for desired time intervals.

2.5. Virus Recovery from cRNA and Detect the Presence of Virus by AC-ELISA, RT-PCR and Plaque Assay

Two days after transfection, cells were frozen -thawed and centrifuged at 700 g to remove cellular debris. The supernatant was passaged for 4 times in the primary bursal cells, harvesting the cells for ELISA. In order to screen the recombinant virus from many samples AC-ELISA was performed. Each well of 96-wells polystyrene ELISA plates (Costar, Cambridge, MA, USA) were coated with 100 μl of chicken polyclonal IBDV antiserum, diluted in PBS at a ratio of 1:4000. After incubation at 37℃ for 1 hour, the plate was washed three times with washing buffer (1% Tween 80 in PBS) and each well was blocked by 100 μl of blocking buffer (0.5% gelatin in PBS) at 37℃ for 0.5 h. After three washes of the plate with washing buffer, 100 μl sample including positive and negative control was added in duplicate. The plate was then incubated at room temperature for 1 h and washed with washing buffer before 50 μl of MAbs M6 or B29 [25,26]., diluted 1:2500 and 1:1000 in antibody diluent (5% NaCl and 4% BSA in washing buffer) respectively, were added to the wells in duplicate. After incubation for 1 h at room temperature, the plate was washed three times with washing buffer. Subsequently, 50 μl of goat anti-mouse IgG-horseradish peroxidase (Sigma) diluted 1:1000 with antibody diluent was added. One hour later at room temperature, the plate was washed three times with washing buffer. After addition 100 μl TMB peroxidase substrate (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD, USA) and incubated at 37℃ for 15 min, the reaction was stopped by adding 100 μl 1 M H_3PO_4. The result was read by an ELISA reader at the optical density at 450 nm (OD450). If OD value of sample is greater than mean OD value plus 3 times standard deviation of negative control sample, then the sample is considered as positive and was stocked at −86℃ for future use.

The titre of virus mutants was determined using plaque assay [27] and prepared for future animal experiment. The titre is represented as PFU/ml.

IBDV mutants were reversely transcribed using outside 1 and nested PCR was amplified using outside 1, outside 2, inside 1 and inside 2 primer (**Table 2**).

2.6. Genetic Stability Analysis

If changes in the amino acid sequence occurred during passaging viral RNA of IBDV before challenge, the identity of virus have to be confirmed. The virus mutants were subjected to RT-PCR using oligonucleotides outside 1 and outside 2 for IBDV with VP2 mutation, VP3 outupper and VP3 outlower for IBDV with VP3 muatation before challenge (**Table 2**). Nested PCR was ampli-

fied with 783 inupper and 62, 21 inlower to identify virus with VP3 alteration (**Table 2**). Cloned PCR fragments of IBDV mutants were sequenced and obtained sequences were analyzed with DNAStar.

2.7. Virulence of IBDV Mutants in Young SPF Chickens

Forty eight 4-week-old SPF White Leghorn chickens were divided randomly into six groups including positive control group. Chickens were infected via eye and nose drop with total 1200 PFU. Non-inoculated hatchmates were used as negative controls. During the course of the experiment animals were observed daily for clinical signs and mortality. At 4 days p.i., all alive chickens from each group were bled and euthanized. The bursa of each chicken (include alive and dead) was removed, weighed and subdivided into two parts. One part was used for detecting the presence of IBD viral antigen by means of an AC-ELISA and RT-PCR. The second part was fixed in 10% neutral-buffered formalin for histology. Formalin-fixed bursal samples were embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E). Microscopic bursal lesion score (BLS) was determined by histopathological analysis of the bursa. BLS was evaluated on a scale of 0 to 5 as follows: 0, no abnormalities; 1, 1-20%; 2, 21-40%; 3, 41-60%; 4, 61-80%; and 5, 81-100% lymphocyte depletion [28].

2.8. Detection of Viral Antigen in Bursae after Challenge

Bursae were homogenized with homogenizer. The presence of virus in the bursal homogenate was detected with AC-ELISA which incorporated Mab 6 recognizing VP2-located epitopes [25].

3. RESULTS

3.1. Determination of Nucleotide Sequence of Harbin-1 Mutant

To establish a reverse genetics system the complete genomic sequence of Harbin-1 mutants was determined. The mutagenized plasmids were obtained with the alteration of two amino acids, Q253H-A284T, H783Q-V862M, V862M-I921V, H783Q-I921V.

3.2. Rescue of Recombinant Virus from cDNA

Primary bursal cells were transfected with synthesized cRNA of mutated segment A and intact segment B by means of lipofectin (Invitrogen). After every transfection, the resultant supernatant was used for RT-PCR and AC-ELISA to detect the presence of viruses. The samples were performed to RT-PCR after IBDV antigen was detectable using AC-ELISA. Electrophoresis result showed that there is one 209 bp band, whose sequence located in VP2 hypervarible region, on 1.2% agarose gel. The result of RT-PCR and AC-ELISA demonstrated that virus mutants were successfully recovered. From 10 transfection samples we obtained four mutant viruses designated as $H_{253/284}$, $H_{783/862}$, $H_{862/921}$, $H_{921/783}$ and rescued Harbin-1 named rHarbin-1.

3.3. Genetic Stability Analysis

Sequence analysis of the RT-PCR products confirmed the identity of the IBDV used. No amino acid substitutions compared to the sequence of the used plasmids (p253/284 m, p783/862 m, p861/921 m, p921/783 m) were found within the region flanked by primers used for RT-PCR, proving the genetic stability of the virus during virus pass aging.

3.4. Virulence Determinants for VP2 and VP3 in Chinese vvIBDV Strain

To evaluate the virulence of all virus mutants animal experiments were performed. Animals infected with vvIBDV (rHarbin-1) and $H_{783/862}$ showed severe clinical signs of IBD. The mortality rates were 7/8 for rHarbin-1, 5/8 for $H_{783/862}$, 1/8 for $H_{253/284}$ and $H_{862/921}$. In contrast, none of the animals infected with $H_{921/783}$ died or showed clinical signs of IBD. Bursae of chickens infected with the different virus mutants showed depletion of bursal cells in lymph nodule with remarkable differences (**Figure 1**). rHarbin-1 and $H_{253/284}$, $H_{783/862}$ induced severe bursal lesion (BLS of 5, 1.6, 3.1 respectively); $H_{862/921}$ induced mild lesion (BLS 1.5); $H_{921/783}$ hardly induce lesion (BLS 0). As to the ration of bursal weight and body weight, rHarbin-1 and $H_{783/862}$ showed severe bursal atrophy (3.39, 3.94 respectively). There was no remarkable difference among $H_{253/284}$, $H_{862/921}$, $H_{921/783}$ and negative control (4.0, 4.8, 4.94, 4.71 respectively) (**Table 3**). The above-mentioned results demonstrated that V862 and I921 in VP3 are probably the major virulence determinants, furthermore, 862 and 921 in VP3 has the stronger ability to manage virulence than 253 and 284 in VP2.

4. DISCUSSION

In recent years, many investigators have shown that mutations in the viral genome often lead to changes in the virulence, pathogenesis of animal viruses. A single amino acid substitution in the West Nile Virus Nonstructural protein NS2A disables its ability to inhibit Alpha/Beta interferon induction and attenuates virus in mice [18]; point mutations in an infectious bovine viral diarrhoea virus type2 cDNA transcript yields an attenu-

Table 3. Results of chicken challenged by four recombinant viruses.

Virus	Number of Chickens*	Mortality	Avg bursa/body wt, Ratio (SD), 10^3	Avg BLS** (SD)	Pathological Lesions
$H_{253/284}$	8	1/8	4.0 (2.0)^{ab}	1.6 (1.1)^{b}	individual lymphatic nodule necrosis and atrophy in dead chicken
$H_{783/862}$	8	5/8	3.9 (1.5)^{ab}	3.1 (2.6)^{c}	lymphatic nodule severe necrosis and atrophy in dead chickens
$H_{862/921}$	8	1/8	4.8 (1.4)^{b}	1.5 (1.4)^{b}	lymphatic nodule partly necrosis and slightly atrophy in dead chicken
$H_{921/783}$	8	0/8	4.9 (0.9)^{b}	0 (0.0)^{a}	lymphatic nodule slightly atrophy and widen interstice close to normal
rHarbin-1	8	7/8	3.4 (0.9)^{a}	5 (0.0)^{d}	lymphatic nodule appear necrosis, congest and hemorrhage
Native Harbin-1	8	7/8	3.1 (0.4)^{a}	5 (0.0)^{d}	lymphatic nodule appear necrosis, congest and severe hemorrhage, atrophy
Negative control	8	0/8	4.7 (0.3)^{b}	0 (0.0)^{a}	normal

*The indicated number of 4-week-old SPF chickens were infected via the eye and nose drop; **BLS of BF of each chicken investigated. Values within the same row with the same superscript letters are not significant ($P < 0.05$).

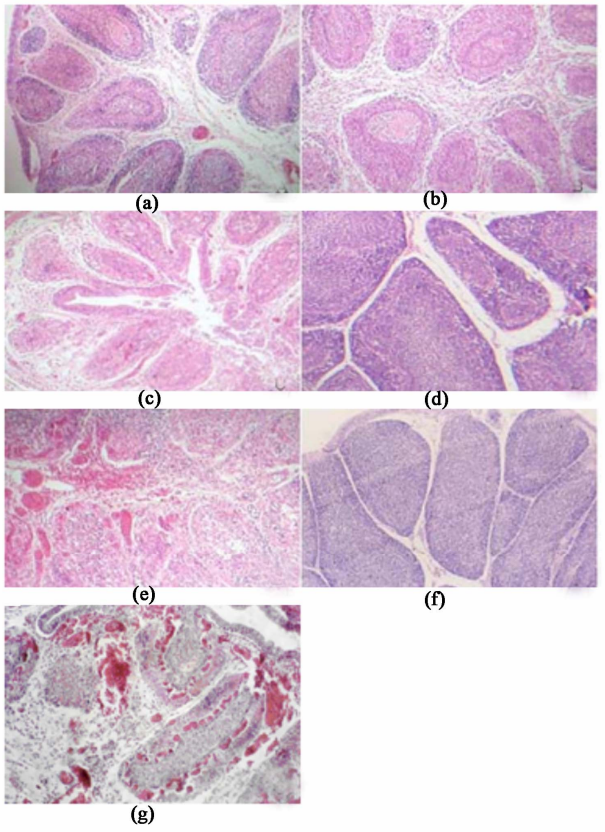

Figure 1. Microscopic pathological effect in bursae challenged by virus mutants (10 × 20) (a) $H_{253/284}$ single lymph nodule necrosis, atrophy, BLS 4; (b) $H_{783/862}$ lymph nodule severe necrosis, atrophy, BLS 4.5; (c) $H_{862/921}$ lymph nodule partly necrotize, BLS 2.3; (d) $H_{921/783}$ close to normal, BLS 0; (e) rHarbin-1 lymph nodule necrosis, congest and hemorrhage BLS 5; (f) CK (negative); (g) Harbin-1 (positive control) lymph nodule necrosis, congest and severe hemorrhage, atrophy BLS 5.

ated and protective viral progeny. Virulence of swine vesicular disease virus is determined at two amino acids in capsid protein VP1 and 2A protease [14]. Above mentioned phenomena elicit researchers on IBDV and they dedicated to study the virulence mechanism. A number of researchers such as Brandt, Yamaguchi, Lim, Mundt and so forth assumed position 253, 279, 284 amino acids in VP2 hypervarible region control phenotype, and could bind with B lymphocyte [3,17,21,29]. Lots of evidence showed that hypervarible region in VP2 involved in conformation dependent epitope and stimulate the chicken to produce protective neutralizing antibody [10, 29].

The result of chickens challenged with viruses showed that $H_{253/284}$ could induced slighter lesion than parental virus vvIBDV (Harbin-1), but in $H_{783/862}$ group, there are two kinds of appearance ,the bursa in alive chickens had not showed pathological sign, which could be due to the individual difference, but the bursae of dead chickens showed severe necrosis and atrophy, B lymphocyte depletion was up to the same 80-90% as Harbin-1; in $H_{862/921}$ group, a bursa of dead chicken had the same pathological lesion as Harbin-1, lymphocyte depletion up to above 90%, in other bursae of alive chickens depletion is only 10-20%, and appear partly necrosis and atrophy; in $H_{921/783}$ group bursae had very slightly pathological lesion except minor widening interstice, suggesting bursa was slight swollen. Therefore, $H_{921/783}$ virus appeared the slightest pathological lesion among all virus mutants. Compared with mDT-VP3C and mDCT-VP3C rescued by Boot who substituted the C-terminal part of VP3 of serotype 1 vvIBDV (isolate D6948) for the corresponding part of serotype 2 IBDV [22], $H_{921/783}$ induced slighter pathological lesion than

mDT-VP3C and mDCT-VP3C. mDT-VP3C and mDCT-VP3C could induced same bursa lesion as wild type D6948 and rD6948, suggesting mDT-VP3C and mDCT-VP3C had stronger residential virulence, but $H_{921/783}$ virus hardly has no residential virulence.

Our experiment demonstrated that VP3 and VP2 contain the determinant for virulence too besides VP2 in one strain. However, up to now most researches assume VP2 play an important role in virulence. The reason for this paradox about virulence controlling mechanism is unknown. Molecular determinant of virulence may depend the strains used. In addition we used two alterations of amino acid in this paper. Single alterations of aa 783, 862 and 921 were not tested, further study may be necessary to identify if single amino acid function or both of them function in virulence at the same time.

5. CONCLUSIONS

V862, I921 in VP3 is obvious virulence marker however I921 has more potential ability to control virulence than V862 and H783. Through animal challenge test we make clear the site in VP2 and VP3 involved in virulence, furthermore, the ability of 862 and 921 to control virulence in VP3 is more powerful than 253 and 284 in VP2.

6. ACKNOWLEDGEMENTS

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