

# A simple and efficient method to rescue very virulent infectious bursal disease virus using SPF chickens

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**Abstract** Reverse genetic systems for efficient generation of very virulent infectious bursal disease virus (vvIBDV) are currently limited. In this study, we have developed a simple and efficient way to rescue vvIBDV using SPF chickens. The genome of a vvIBDV strain, HLJ0504, flanked by hammerhead and hepatitis delta ribozyme sequences, was cloned downstream of the cytomegalovirus enhancer and the chicken beta-actin promoter of the vector pCAGGS. After transfection of DF-1 cells, cell suspensions were injected into the bursa organ of three-week-old SPF chickens. Using this system, vvIBDV was recovered at high titers after one passage, and the rescued vvIBDV remained highly lethal to SPF chickens. This simple and efficient method to rescue vvIBDV will be a valuable tool for better understanding the molecular virulence determinants of vvIBDV.

## Introduction

Infectious bursal disease virus (IBDV) is a member of the family *Birnaviridae* and is the causative agent of infectious bursal disease (IBD), which has a major economic impact on the poultry industry. The genome of IBDV consists of two double-stranded RNA segments designated segment A and segment B. The larger segment A encodes a polyprotein that is cleaved to produce mature viral proteins VP2, VP3 and VP4. A second open reading frame, which

precedes and overlaps the polyprotein gene, encodes the nonstructural protein VP5. The smaller segment B encodes VP1, a multifunctional protein with enzymatic activities.

The emergence of IBDV with enhanced virulence, known as very virulent IBDV (vvIBDV), has stimulated research on IBD and IBDV [7]. vvIBDV has spread worldwide and has brought new challenges for the prevention and control of the disease. Several studies have aimed to identify the molecular determinants of the enhanced virulence of vvIBDV, but to date, these determinants have not been fully defined [2, 4].

vvIBDV replicates specifically in developing B-lymphoid cells in the bursa of Fabricius of poultry. Adaptation of vvIBDV to cell culture is always correlated with attenuation [11]. Several reports have described the amino acid changes in VP2 that contribute to cell culture adaptation and viral attenuation [5, 10]. To define virulence determinants in other genes, reverse genetic systems to easily generate vvIBDV are needed. Different reverse genetic systems for the generation of IBDV have been established and used [1, 8, 10]. Generally, after transfection in a suitable cell line, the lysates are passaged in chicken embryo fibroblast (CEF) cells, and after several blind passages, the rescued viruses are harvested. However, established reverse genetics systems are seldom used to generate vvIBDV because of the limitations of replicating vvIBDV in cell culture, and therefore very few studies have been based on the rescue of vvIBDV. These limitations have greatly hampered research towards understanding the molecular pathogenesis of vvIBDV and vaccine development. vvIBDV cannot be recovered using rescuing systems that require direct transfection of cell lines. There is an urgent need to develop a new and efficient method for vvIBDV rescue. In this study, we describe a simple way to rescue vvIBDV with a high efficiency. The method will be of value for investigating the

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molecular determinants that enhance virulence in vvIBDV and could facilitate studies of pathogenesis of vvIBDV.

## Materials and methods

### Virus, cells and plasmids

An vvIBDV strain, HLJ0504, was isolated from the field and preserved in our laboratory. DF-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in a humidified incubator with 5% CO<sub>2</sub>. The plasmids pT-HLJ0504A and pT-HLJ0504B, containing segment A and B of HLJ0504, respectively, were constructed previously. The eukaryotic expression vector pCAGGS was kindly provided by Dr. J. Miyazaki, University of Tokyo, Tokyo, Japan.

### Chickens

Specific-pathogen-free (SPF) chickens were provided by Harbin Veterinary Research Institute and housed in negative-pressure, filtered-air isolators. All animal experiments were approved by the Animal Ethics Committee of the institute.

### Construction of plasmids

We used a previously established RNA polymerase II reverse genetics system [9]. The segments A and B of HLJ0504 were flanked by hammerhead ribozyme (HamRz) and hepatitis delta ribozyme (HdvRz) sequences downstream of the CMV enhancer and chicken beta-actin promoter. The HamRz and HdvRz cDNA sequences were fused with segment A or segment B of HLJ0504 as described previously [10]. The resultant plasmids were designated pCAGGS-HLJ0504A and pCAGGS-HLJ0504B, respectively.

### Virus rescue

DF-1 cells were grown in six-well cell culture plates to 80% confluence and were co-transfected with 1.5 µg

pCAGGS-HLJ0504A and 1.5 µg pCAGGS-HLJ0504B using Invitrogen Lipofectamine<sup>TM</sup> LTX reagent. The ratio of plasmids and LTX reagent was optimized (data not shown), and the ratio 1:5 provided the highest transfection efficiency. Briefly, 3 µg plasmid DNA was mixed with 3 µl reagent in 500 µl Opti-MEM and incubated at room temperature for 5 min, and 15 µl LTX reagent was then added to the mixture and incubated for an additional 30 min. After removing the DMEM from cells and replacing it with fresh Opti-MEM, the DNA mixture was added gently to the dish and incubated at 37°C. Four to 6 h later, the cells were washed with Opti-MEM and maintained in DMEM supplemented with 10% FBS for another 48 h. After the 48-h incubation, the cells were frozen and thawed three times, and 1 ml of the resultant cell suspensions were injected into the bursa organs of three three-week-old SPF chickens from the two sides of the tail on the body surface using 2-ml injectors. The inoculation depth was about 1 cm, and the inoculation angle was between 45° to 90° from the two sides. PBS was used as a negative control.

### Identification of the rescued virus

Three days after injection, we excised and dissected the bursa tissues from all injected chickens, both those that survived and those that did not, and examined them for other signs of viral infection. The rescued virus was named rvH-4B. A portion of the bursa tissue was fixed by immersion in 10% neutral buffered formalin for histopathology. Another portion was used for RNA isolation and for RT-PCR amplification targeting both genome segments. The primers used for identification of the rescued virus are listed in Table 1. The remaining part of the bursa was used for electron microscopy.

### Rescued virus titration

Bursal tissue (0.1 g) was homogenized in 500 µl PBS and filtered through a 0.22-µm-pore-size filter. Nine-day-old SPF embryonated eggs were inoculated with 100 µl processed bursa suspension via the chorioallantoic membrane (CAM). The titer of the processed bursa suspension was determined as ELD<sub>50</sub> per milliliter using the Reed-Muench formula.

**Table 1** Nucleotide sequences and positions on genomic segments of the primers used in this study

Primer designation	Sequence	Nucleotide no.
A836-up	5'-GCCATCACAAGCCTCAGCAT-3'	836-856 of segment A
A1101-low	5'-ACTTGCTGACCATGACATCT-3'	1080-1101 of segment A
B85-up	5'-GCTCCTCTTCTTGATGATTCT-3'	85-106 of segment B
B1040-low	5'-GCCCGCATGATAAGTACCAAT-3'	1019-1040 of segment B
B1573-up	5'-GCCACATTCATCAACAACCAT-3'	1573-1594 of segment B
B2288-low	5'-ACTCAGTCCGGCTTCATTCC-3'	2267-2288 of segment B

## Chicken experiment

Ninety three-week-old SPF chickens were randomly divided into three groups. Challenged group 1 ( $n = 30$ ) was inoculated via the ocular and intranasal routes with  $10^3$  ELD<sub>50</sub> of rvH-4B, challenged group 2 ( $n = 30$ ) with  $10^3$  ELD<sub>50</sub> of the field isolate strain HLJ0504, and the control group ( $n = 30$ ) with PBS. Chickens were observed for a period of seven days to evaluate the mortality rate.

## Results

### Detection of the recovered virus

Three days post-infection, two out of three chickens injected with transfected cell suspensions died. Dissected bursae of all chickens injected with transfected cell suspensions showed signs of hemorrhage and inflammation (Fig. 1a) while the bursae of chickens injected with PBS appeared normal (Fig. 1b). Histopathological examination of the bursae from chickens injected with transfected cell suspensions showed severe bursa lesions with B lymphocyte necrosis and depletion of follicles and proliferation of connective tissue (Fig. 2a). The bursae from chickens injected with PBS showed no signs of disease (Fig. 2b). In addition, electron microscopy revealed an abundance of IBDV particles in the bursa tissue of chickens injected with transfected DF-1 cells (Fig. 3). The virions were non-enveloped and about 60 nm in diameter.

### RT-PCR detection

To determine if IBDV RNA was present in the bursae of chickens injected with transfected DF-1 cells, we isolated total RNA from a section of bursa and subjected it to

analysis by RT-PCR. Using primers specific for both segments of IBDV, we observed the expected amplification fragments in the bursae of chickens injected with the cell suspensions but not in bursae of chickens injected with PBS or when M-MLV reverse transcriptase was omitted (data not shown). Sequencing of the RT-PCR products indicated that the rescued virus was derived from the plasmids used to transfect DF-1 cells prior to injection into SPF chickens.

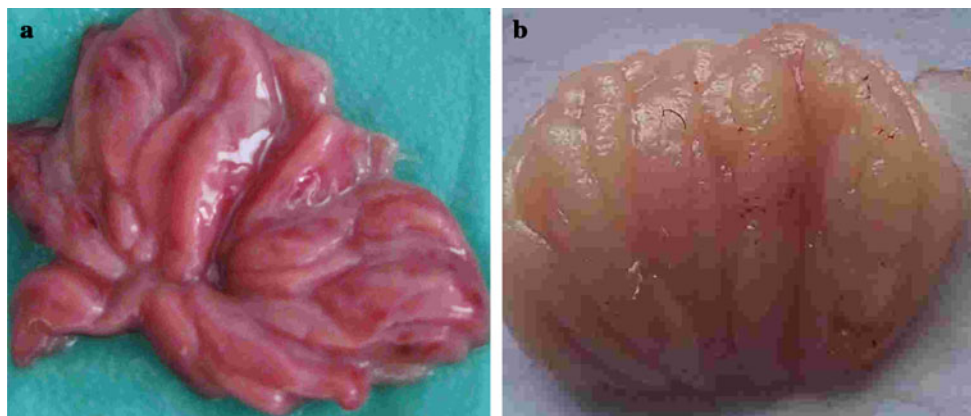
### Pathogenicity

Three days postinfection, chickens in the challenged groups 1 and 2 began to die, and those that survived had clinical symptoms including depression, diarrhea and dehydration. At seven days post-injection, the mortality rate of the challenged group 1 was 80%, and the mortality rate of the challenged group 2 was 73.3%, while all of the chickens in the control group remained healthy.

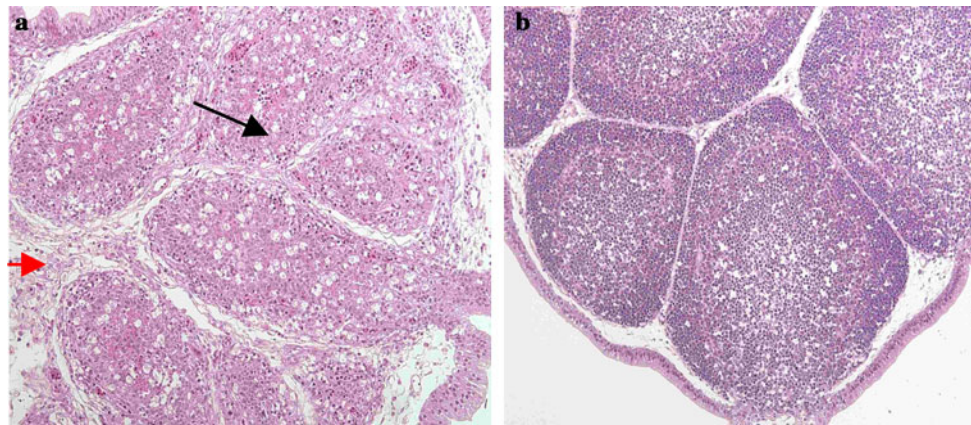
## Discussion

vvIBDV strains are unable to grow on non-B lymphoid cells, and until recently, most IBDV generated by reverse genetics were cell-culture-adapted viruses. The lack of an efficient method to rescue vvIBDV has greatly hampered studies of pathogenesis of vvIBDV.

Previous methods of rescuing vvIBDV mostly applied the strategy of inoculating the CAM or primary bursal cells with cell suspensions after transfection in cell lines [2, 3, 6]. Boot et al. applied an *in vivo* T7 expression reverse genetic system to rescue very virulent strain D6948. After transfection in QM5 cells, primary bursa cells were used for viral propagation. They claimed that vvIBDV could only infect some B lymphoid cells, but no destruction of

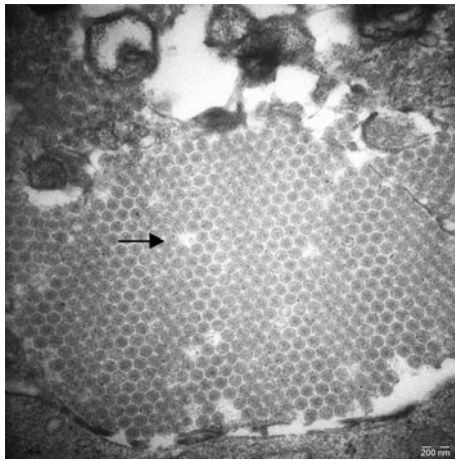


**Fig. 1** Bursal samples from chickens inoculated with cell suspension transfected with plasmids derived from the HLJ0504 (a) or PBS as a negative control (b). Hemorrhage and inflammation can be observed in (a)



**Fig. 2** Histopathological examination of bursal samples. Bursal sample from chickens inoculated with transfected cell suspension (a) or PBS (b). B-lymphocyte necrosis and depletion in follicles (dark

arrow) and proliferation of connective tissue (red arrow) can be observed in (a). (magnification  $\times 20$ ) (color figure online)



**Fig. 3** Electron microscopy of rescued virus in bursal tissue. Virus particles (dark arrow) with a diameter of about 60 nm were observed in the tissue. (Scale bar, 200 nm)

the monolayer could be observed, so it was impossible to monitor CPE. We also tried to use primary bursa cells for propagation of the vvIBDV virus. We first tried to infect primary bursa cells with the vvIBDV field isolate strain HLJ0504, but we were unable to detect viral replication by immunofluorescence (data not shown). Brandt et al. used a cRNA-based reverse genetic system to generate a chimeric virus that contained the VP2 of a virulent strain. After transfection of Vero or CEF cells via electroporation, the virus was recovered after propagation in the CAM. However, some vvIBDV strains could not be recovered using this strategy [6]. In a previous experiment, we tried to use CAM inoculation after transfection of DF-1 cells to generate vvIBDV but were unable to recover viable vvIBDV even after three blind passages. In this study, the first passage of the transfected cell suspensions in SPF chickens lead to death, and all of the chickens (3/3) were infected

with vvIBDV based on examination of bursa damage combined with RT-PCR and electron microscopy. We determined the ELD<sub>50</sub> titer of the recovered virus in embryonated eggs, and it was as high as  $10^{6.5}$  ELD<sub>50</sub> per milliliter. Additionally, we generated vvIBDVs for another study that had mutations in the VP1 protein (data not shown), and the rescue efficiencies were similar to those reported here. The rescue efficiency was so high that the viruses could be harvested after the first passage in SPF chickens without any blind passages. The sequences of both segments remained the same as the parental field isolate. The mortality rate of chickens infected with the rescued virus rvH-4B remained as high as 80%, which is similar to the parental field isolate strain HLJ0504, which had a mortality rate of 73.3%. To generate vvIBDV in this study, we used a RNA polymerase IIb-based reverse genetics system which was previously shown to be effective in generating the cell-culture-adapted virus Gt. Our results confirmed that this system was also suitable for rescuing vvIBDV strains.

The method used to inoculate chickens was important for achieving the high rescue efficiency obtained. In previous studies, we attempted to inoculate three-week-old chickens with the transfected cell suspension via the ocular and intranasal routes but failed to recover infectious virus. Since the bursa tissue is the target organ of IBDV, and vvIBDV replicates efficiently in the bursa of Fabricius, bursa injection of SPF chickens was effective in the rescue of vvIBDV. The location of the bursa is near the body surface, making it easy to inject cell suspensions into the bursa tissue from the back of the chickens. This study confirms that it is possible to rescue vvIBDV from chickens through injection of transfected cell suspensions into the bursa tissue. Three- to six-week-old chickens are susceptible to IBDV, so we chose chickens at this age for recovering vvIBDV.



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